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13. ABSTRACT *(Maximum 200 Words)*

We previously demonstrated elevated HGF and Met expression in regions of invasive human breast carcinomas, suggesting a role of autocrine HGF loops in invasive breast cancer. As a first step to validate this hypothesis, we have now demonstrated that: a) elevated HGF and Met expression occurs in some newly-derived breast and non-small cell lung carcinoma cell lines; and b) several carcinoma cell lines showed autocrine tyrosine-phosphorylation of Met and increased cell survival, consistent with the presence of autocrine HGF loops. Thus, we have identified autocrine HGF loops in newly-derived breast carcinoma and NSCLC cell lines, and have correlated the activation of HGF autocrine loops with cell survival. The next stage of this study is to design peptide antagonists of HGF that block HGF ligand receptor binding, and to test the efficacy of these antagonists in inhibiting tumorigenic functions. This information will be important in the search for novel targets for therapeutic intervention in breast cancer.

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INTRODUCTION:

Role of HGF in mammary tumor progression: Expression of HGF in nonmalignant epithelium is generally under tight negative regulation (1). Paracrine stimulation of normal breast epithelium with HGF, in co-operation with other growth factors (e.g., neuregulin), promotes branching morphogenesis (2). However, HGF (3-5) and its receptor, Met (6), are frequently over-expressed in breast cancer as well as many other cancer types. This high level of HGF and Met expression has been identified as a possible independent predictor of poor survival in breast cancer patients (6). Recent findings from our laboratory show that invasive human carcinoma cells co-express HGF and Met, particularly at the migrating tumor front (5). Furthermore, expression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (7,8,9) or in transformed cell lines (10,11,12) promotes tumorigenesis and metastasis. During tumorigenesis, HGF stimulates loss of epithelial differentiation (13), increased cell survival (14,15), invasion (16), and metastasis (10; 17). These observations have suggested that sustained activation of the Met signal transduction pathway may be important for development of cancers through autocrine or paracrine mechanisms.

Role of c-Src in malignancy: A number of signalling molecules, such as c-Src (23), Ras (18), Grb2 (19) and phosphatidylinositol (PI) 3-kinase (20), have been shown to be part of the HGF/Met signalling pathway. The c-Src non-receptor tyrosine kinase is expressed in many cell types, and is activated in response to HGF and binding to Met (21-23). Activation of c-Src kinase is required for cell spreading and cell motility, and can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (VEGF) (24) and parathyroid hormone-related peptide (25). Increased activation of the tyrosine kinase c-Src occurs in many cancer cells, and c-Src plays a critical role in breast cancer. In a transgenic mouse model, over-expression of an activated form of c-Src induces mammary hyperplasia (26). Furthermore, c-Src kinase is activated during polyoma middle T-induced mammary tumorigenesis in transgenic mice. However, expression of polyoma middle T in *c-src*$^{-/-}$ background mice fails to cause tumor formation (27). Together, these results show that c-Src is necessary, but not sufficient, for mammary tumorigenesis.

Structure, maturation and isoforms of HGF: Mature HGF protein contains a heparin binding domain, followed by four kringle-like domains designated K1 to K4, as well as a C-terminal region homologous to serine protease but lacking any protease activity (28). In human, HGF is first produced as a single polypeptide precursor of 728 amino acids. Following removal of the N-terminal 31 amino acids, this pro-hormone form is secreted and then cleaved by an unknown serine protease to form the active mature HGF (28). The cleavage at the Arg^{494}-Val^{495} site is essential for HGF activity and several serine proteases have been shown to process pro-HGF at this site *in vitro* (30). Other naturally occurring forms of HGF are also found *in vivo*. Truncated versions of HGF derived from alternative mRNA splicing can act either antagonistically or agonistically towards native HGF. NK1 which contains all the N-terminal amino acids up to K1 can bind to Met and acts in both agonistic or antagonistic manners to HGF depending on culture conditions (31-33). NK2, containing the HGF sequence up to K2, acts mainly in an antagonistic manner (34). The presence of these isoforms in nature may provide a subtle way of regulating HGF activity *in vivo*.

HYPOTHESIS AND OBJECTIVES: Whereas nonmalignant epithelial cells normally do not express HGF, our laboratory (5), and others (35,36), have recently demonstrated co-expression of HGF and Met mRNA in primary human breast carcinomas, as well as in regions of benign ductal hyperplasia. Based on these findings, our **hypothesis** is that co-expression of HGF and Met results in the establishment of an autocrine HGF loop and sustained activation of Met, which acts perhaps with other signalling pathways as a selective advantage for autonomous growth and metastasis of mammary carcinoma cells. De-regulation of expression of functionally-active HGF in mammary epithelial cells could occur at several levels from transcription (1,29) to post-translational modification by enzymatic processing (28,30). The following **ORIGINAL OBJECTIVES** were proposed in this grant:

I) **To assess secretion and maturation of HGF and Met expression in nonmalignant and malignant breast epithelial cells, and to correlate with HGF-induced cellular functions;**

II) **To examine the regulation of HGF mRNA expression and maturation of HGF protein in nonmalignant and malignant mammary epithelial cells *in vitro* and *in vivo*; and**

III) **To determine if up-regulating or down-regulating HGF mRNA and protein expression in nonmalignant and malignant mammary epithelial cells affects the transformed and tumorigenic phenotypes of these cells *in vitro* or *in vivo* (modified based on new information from Objectives I and II).**

MATERIALS AND METHODS: Breast carcinoma cell lines used in this study are described in Table I. Details of materials and methods are described in publications referenced in the Results section. See also Figure legends.

PROGRESS AND RESULTS:

Objective (I): To assess secretion and maturation of HGF and Met expression in nonmalignant and malignant breast epithelial cells, and to correlate with HGF-induced cellular functions:

Using *in situ* hybridization, we (5) and other laboratories (35) have shown that HGF mRNA is expressed in human invasive breast carcinoma cells as well as in regions of ductal epithelial hyperplasia. Preliminary results also showed immunoreactive HGF protein associated with carcinoma cells in human breast tissues (Tuck *et al.*, unpublished result). However, it is not known from these studies whether post-translational processing of pro-HGF, required for HGF activity, occurs in benign or malignant epithelial cells from primary breast tissues. Since transcriptional regulation of HGF in normal epithelium is under tight negative regulation (1), it was important to determine whether functionally active HGF protein was produced by malignant carcinoma cells. We therefore examined the expression and activity of HGF and Met in newly-established nonmalignant and malignant breast epithelial cell lines.

a) Detection of HGF mRNA and protein: To detect expression of HGF mRNA in nonmalignant and malignant breast epithelial cells, we used RT-PCR analysis. Primers were designed to detect HGF cDNA, and to recognize the corresponding cDNAs of mouse and human but not the homologous family members MSP (Fig. 1). Primers specific for the house-keeping gene, β-glucuronidase (GUS B, 41), were used as an internal control. A linear range of amplification was found between 10 and 30 cycles for HGF and GUS B. Similarly, the product amplification was directly proportional to the amount of template used (data not shown).

To aid the studies of HGF protein, we used a copper (II) affinity chromatography technique to isolate HGF from conditioned media of cell lines, developed in my laboratory (42). The principal of separation of HGF from biological samples by Cu (II) affinity chromatography is based on the fact that HGF has several cationic sequences (His-X-His) in the kringle domains of the HGF molecule. Greater than 70% of the HGF protein bound to the copper (II) column was eluted with 80 mM imidazole, as determined by western blotting.

b) Expression of HGF and Met mRNA and protein in newly established human breast carcinoma and non-small cell lung carcinoma cell lines: Our results showed that 12/12 mammary epithelial and carcinoma cell lines expressed Met mRNA, and all but one carcinoma cell line (WO-E) expressed Met protein (Table I). In addition, HGF mRNA was expressed in 8/12 mammary epithelial and carcinoma cell lines tested, and five cell lines tested expressed HGF protein, detected by western blotting (Fig. 2A & Table I). One breast carcinoma cell line, MCF10A1T3B, showed several immunoreactive lower molecular weight bands, which were not present in the corresponding non-malignant cell line MCF10A1 or another carcinoma cell line, EL-E. The possible function of the observed lower molecular weight bands in CM from MCF10A1T3B cells is currently being examined (see below).

Recently, non-small cell lung carcinomas (NSCLC) have been shown to express HGF mRNA and protein (75). Therefore, to provide a comparison with another carcinoma cell type, we have also examined HGF and Met expression in a series of NSCLC cell lines. Our results, summarized in Table II, showed that 2/6 NSCLC cell lines expressed Met protein. In addition, 4/6 NSCLC cell lines expressed HGF mRNA and protein (Fig. 3A), two of which also expressed Met. One nonmalignant human bronchioepithelial cell line (HBE) (44) showed no detectable PCR product for HGF and was used as a baseline control for comparison with other cell lines (App. III and Table II).

c) Activity of putative HGF protein secreted by mammary and NSCLC carcinoma cell lines: To assess the activity of HGF in CM from carcinoma cell lines, we tested the ability of CMs to activate Met in A549 carcinoma cells (which are Met positive, HGF negative). A549 cells were incubated for 30 min at 37°C with CMs, and lysed. Cell lysates were immunoprecipitated with anti-Met IgG, and subjected to SDS-PAGE (reducing conditions) and western blotting with anti-phosphotyrosine antibody to assess the tyrosine phosphorylation status of Met (Fig. 2B). A control showed strong tyrosine phosphorylation of Met in A549 cells incubated with HGF (40 ng/ml), compared to A549 cells incubated alone. The results showed that CM from EL-E, MCF10A1 and MCF10A1T3B cell lines stimulated tyrosine-phosphorylation of Met in A549 cells. In addition, 3/4 HGF-producing NSCLC cell lines showed activity in the Met-phosphorylation assay (Fig. 3B). Interestingly, CM collected from MCF10A1T3 B cells induces tyrosine-phosphorylation of Met in A549 cells to a

lesser extent than CM from EL-E cells, suggesting that there is less active HGF in MCF10A1T3B CM. In contrast, CM from WO-E cells, which showed no HGF protein, had no effect. These results indicate that the majority of breast carcinoma and NSCLC cell lines secrete active HGF (See Table II and App. II).

d) Examination of Met activation status in breast carcinoma and NSCLC cell lines: A prediction from the above studies is that carcinoma cell lines that express both active HGF and Met would show constitutive activation of Met, consistent with the establishment of an autocrine HGF loop in these cells. To test this possibility, we have examined the tyrosine-phosphorylation level of Met in carcinoma cell lines, using western blot analysis. One mouse carcinoma cell line showed co-expression of HGF and tyrosine-phosphorylated Met, and spontaneous invasion through Matrigel (37). None of the human breast carcinoma cell lines tested showed autocrine tyrosine-phosphorylation of Met, although addition of exogenous HGF stimulated tyrosine-phosphorylation of Met in all cases (data not shown). However, the possibility that autocrine activity of HGF is inhibited by ligand degradation, or association with proteoglycans (e.g. heparin) (81), is currently being examined.

Interestingly, two NSCLC cell lines (WT-E and SW900) expressing both HGF and Met showed significant tyrosine-phosphorylation of Met even without treatment with exogenous HGF (Fig. 4). These experiments are consistent with establishment of an autocrine HGF loop in some carcinoma cells co-expressing HGF and Met.

e) DNA synthesis and survival in carcinoma cell lines: To test the biological function of putative paracrine versus autocrine activation of Met in NSCLC cell lines, we examined DNA synthesis and cell survival in various NSCLC cell lines. The base level of DNA synthesis in the absence of exogenous HGF was low in all cell lines, regardless of the level of endogenous HGF produced (Fig. 5C). All Met-positive cell lines required paracrine stimulation with HGF of DNA synthesis, regardless of the level of endogenous HGF produced. A corresponding HGF-dependent increase in ERK1/2 activation was also detected (Fig. 5D). Cell lines which showed no expression of Met did not respond to HGF in the DNA synthesis assay (data not shown).

In contrast, cell lines (SW-900 and WT-E) with autocrine expression of HGF and sustained tyrosine-phosphorylation of Met, consistently showed increased survival under nonadherent serum-starved conditions; whereas A549 cells, which express Met but not HGF, showed a reduced survival response (Fig. 5A). Furthermore, SW-900 and WT-E cells showed sustained high level of tyrosine-phosphorylation of Met under nonadherent conditions compared to A549 cells which did not (Fig. 5B). Thus increased cell survival, but not DNA synthesis, correlated with the expression of HGF and autophosphorylation of Met at tyrosine residues in NSCLC cell lines.

Objective (II): To examine the regulation of HGF mRNA and HGF protein expression in nonmalignant and malignant mammary epithelial cells *in vitro* and *in vivo*:

The results in Objective I imply that at least some carcinoma cell lines express HGF, however the amount and activity of HGF produced in the tumor site, and the stromal effects regulating HGF expression in carcinoma cells are not clearly known. ECM proteins which regulate

a wide range of tissue specific genes, are an important component of mammary stroma (46,47). In this objective, we have therefore focused on: a) the paracrine effect of ECM on the regulation of HGF expression and Met activation, and b) the role of c-Src kinase, which shows increased activity in most breast carcinoma cells and is known to regulate expression of some growth factors, e.g. vascular endothelial growth factor (VEGF) (24).

a) Effect of osteopontin on regulation of HGF expression and cell motility: Bissell *et al.* (46,47) have shown that the basement membrane protein, laminin, promotes morphogenesis and specialized gene expression in normal mammary epithelial cells. In contrast, aberrant expression of ECM proteins is often associated with malignancy. For example, the secreted glycophosphoprotein, osteopontin (OPN), which induces motility of some breast carcinoma cells, has been implicated in malignancy of breast carcinoma (reviewed in Ref. 51). Recently, Tuck *et al.* (51) have shown that expression of OPN is upregulated at the migrating tumor front in invasive human breast cancer. Both OPN and HGF have been shown to stimulate cell migration in a variety of cell types. Therefore, Tuck *et al.* examined the effect of OPN and HGF on migration of breast carcinoma cells at different stages of tumor progression using 21PT (nonmalignant), 21NT (primary tumor), and MDA-MB-435 (metastatic) cell lines (Table I). The results showed that OPN stimulated cell migration, and a synergistic effect with HGF in the migration response was observed (Tuck *et al.*, personal communication).

The above results raise the possibility that OPN may stimulate cell migration at least in part by activation of the HGF/Met pathway. In collaboration with Tuck *et al.*, we have therefore examined the effect of OPN on expression and activity of HGF and Met. We have shown that incubation with OPN was associated with an initial (within 30 min) increase in autoactivation of Met kinase, followed by an increase in expression of Met mRNA (data not shown) and protein (Fig. 6 & 7). The level of OPN-induced Met kinase activity was similar to that observed in response to HGF. These findings suggest that OPN-induced cell migration may act through activation of Met. Experiments are in progress to determine whether OPN affects expression of HGF and autocrine activation of Met in breast carcinoma cells, using approaches described in Objective I. (See App. III).

b-i) **Role of c-Src in regulation of HGF expression in mammary carcinoma cells:** To study the regulation of HGF expression in breast carcinoma cells, we used the mouse mammary carcinoma cell line SP1, which co-expresses HGF and tyrosine-phosphorylated Met (37), and expresses constitutive activation of c-Src kinase (23). Semi-quantitative RT-PCR was performed to determine the levels of *HGF* mRNA in SP1 cells. We first examined the dose-dependent effect of an inhibitor of c-Src family kinases, PP2 (55). Total RNA was isolated from SP1 cells treated with different concentrations of PP2 and used for cDNA synthesis by reverse transcription. Relative *HGF* mRNA levels were determined by RT-PCR using HGF-specific primers, and each sample was normalized to the expression of a house keeping gene β-glucuronidase (GUS B) . The results showed that the PP2 inhibitor reduced *HGF* transcription and mRNA level in a dose-dependent manner to a maximum of 40% of untreated cells (Fig. 8). To assess the effect of c-Src kinase activity on *HGF* mRNA expression, RT-PCR analysis was carried out on RNA extracted from SP1 cells expressing the different c-Src mutants (Fig. 9), or treated with the PP2 inhibitor. Expression of the dominant

negative SRC-RF (K295R, Y527F) mutant or treatment with PP2 reduced the *HGF* mRNA level in SP1 cells by approximately 60%. Conversely, expression of the constitutively active c-Src mutant (SRC-Y527F) increased *HGF* mRNA expression by about two-fold. In a parallel approach, the level of secreted HGF protein was compared in conditioned media collected from the same cells and under the same conditions described in Fig. 10. Expression of the dominant negative SRC-RF mutant or treatment with PP2 significantly decreased the amount of HGF protein secreted by SP1 cells. In contrast, expression of activated c-Src showed an increased amount of secreted HGF protein. Interestingly, cells expressing a constitutively active Src (Y527F) mutant showed increased Met activation compared to untreated SP1 cells, whereas cells expressing a DN SRC-RF mutant showed reduced Met activation (Fig. 11). Together, these data suggest that HGF expression and Met activation are regulated by c-Src kinase activity.

b-ii) A c-Src/Stat3 pathway is required for HGF transcription in carcinoma cells: Based on the above results, we examined the effect of c-Src kinase activity on *HGF* gene transcription using a reporter plasmid. A plasmid containing a 2.7 kb fragment 5' of the *HGF* transcriptional start site ligated to the firefly luciferase gene was transiently transfected into SP1 cells. Bell *et al.* (48) have previously shown that this 2.7 kb fragment of the *HGF* promoter contains all the necessary sequence to direct HGF expression and mimics the expression pattern of the endogenous *HGF* gene in transgenic mice. Transient expression studies in SP1 cells further support the notion that c-Src kinase activity is required for *HGF* transcription. Expression of activated c-Src (SRC Y527F) induces *HGF* promoter activity while expression of the dominant negative c-Src (SRC-RF) reduces *HGF* promoter activity (Fig. 12). To understand the mechanisms regulating HGF expression by c-Src kinase, we carried out a deletion analysis of the HGF promoter and tested for c-Src responsiveness using the *HGF*-luc reporter assay and Src mutants described in Fig. 12. Using this approach, we have located the region responsive to increased c-Src kinase between -274 and -70 bp in the *HGF* promoter (Fig. 12), suggesting that this region is important in c-Src mediated *HGF* transcription. We further showed a co-operative effect between c-Src and Stat3 in enhancing *HGF* promoter activity in SP1 cells (Fig. 13). A similar co-operative effect of c-Src and Stat3 was demonstrated in activation of *HGF* promoter in nonmalignant HC11 epithelial cells, although a greater dependency on c-Src kinase was evident. These results suggest that increased c-Src kinase activity and Stat3 expression can over-ride negative regulation of *HGF* transcription in nonmalignant mammary epithelial cells.

The region which is required for c-Src responsiveness of the *HGF* promoter in breast carcinoma cells contains two putative binding sites for Stat3 protein (Fig. 14). Evidence from other laboratories has shown that Stat3 is activated by v-Src (52,53,54,55), and is required for transformation (53,54). Stat3 is also required for HGF-induced mammary tubulogenesis (56). Although there is no evidence that Stat3 is phosphorylated directly by c-Src, some reports suggest that c-Src and Stat3 interact physically (49). Therefore, it is possible that c-Src regulates Stat3 through tyrosine phosphorylation. Using antibody specific for the tyrosine phosphorylated form of Stat3, we showed that expression of a dominant negative form of c-Src reduced the level of tyrosine phosphorylation of Stat3 in SP1 cells and the expression of a constitutively active c-Src mutant had the opposite effect (App. IV). In addition, we found that the formation of a DNA-protein complex with the two Stat3 binding sites (-110 and -149) in the c-Src responsive elements was dependent on the level of c-Src kinase activity in the cells. Incubation of nuclear extracts from SP1 cells expressing

activated c-Src mutant has more binding activity than that of untransfected SP1 cells. SP1 cells expressing the dominant negative c-Src mutant has the opposite effect. The binding was specific because addition of unlabelled oligonucleotides with unrelated sequence (NS) cannot eliminate the binding while unlabelled oligonucleotides corresponding to the *HGF* promoter Stat3 binding sites (-110 or −149) can efficiently compete for binding (App. IV). Supershift studies using antibodies against specific Stat proteins allowed us to identify Stat3, but not other Stat proteins, as a component of the DNA-protein complex (App. IV). Addition of antibodies against Stat1 (data not shown), Stat5A or Stat5B cannot "supershift" the DNA binding complex. Only anti-Stat3 antibody can further retard the mobility of the DNA-protein complex on the gel, suggesting that Stat3 is part of the DNA-protein complex. Other Stat proteins were ruled out because they were not expressed in mammary tissues. Together, these observations suggest that c-Src kinase may regulate Stat3-dependent transcriptional activation of the *HGF* promoter through direct or indirect tyrosine phosphorylation of Stat3 resulting in increased DNA-binding ability.

c) Role of PI3K: PI3K is a key signalling molecule involved in HGF-dependent cell survival (57), scattering (58) and tubulogenesis (59) of epithelial cells. Recent findings also indicate that PI3K and Akt are recruited to cell-cell junctions (60), and that the PI3K/Akt survival pathway is activated by the formation of E-cadherin-mediated adherens junctions (61). Treatment of SP1 carcinoma cells with the PI3K inhibitor (LY294002, at 10-30 μM) blocks activity of the 2.7 kb *HGF*-luc reporter by up to 60% in a dose-dependent manner (Fig. 15). This inhibition was not affected by truncations of the *HGF* promoter from -2.7 kb to -.5 kb, nor by the Δ1 *HGF* deletion mutant (lacking the c-Src-responsive region (-254 to -70)). To identify the *HGF* promoter region responsive to PI3K, we will carry out a deletion analysis of the −500 bp to −254 bp region, using the *HGF*-luc reporter as readout. Based on these results, further deletions and mutations in the -70 bp to −5 bp region will also be performed. Interestingly, analysis of these regions of the promoter revealed two putative AP1 binding sites (at positions -341 and -62), as well as functional Sp1 (-328) and C/EBP (-12) binding sites (62) (Fig. 14). Considering that PI3K can activate gene transcription through the AP1 transcription factor (63), these sites may be important in PI3K-dependent HGF expression. To corroborate these observations at the functional level, we are testing the effect of constitutively active PI3K mutants (p110CAAX and the p110-activating mutant of Ras V12C40 (64)), and a DN PI3K mutant (Δp85) (65), on *HGF* promoter activity. HGF mRNA, protein expression, Met activation and function will also be assessed in our future studies.

d) Role of Rac1: Recently, Jove *et al.* (66) showed that a Rac1/p38 pathway acts as an intermediate in the activation of Stat3 by c-Src kinase. In addition, Rac1 (and Cdc42) activity is required for cadherin-mediated cell-cell adhesion, and Rac1 acts in co-operation with the MAPK pathway in the HGF-dependent scatter response (67,68). Using activated Rac V14 and RacL61 mutants (from Dr. A. Hall (69)), we have shown that Rac1 strongly stimulates *HGF* transcription (Fig. 16). Co-transfection of Rac V14 with Δ1 *HGF*-luc or 2.7 kb *HGF*-luc showed that 40-50% of Rac1-induced HGF expression required the c-Src/Stat3 responsive region. Experiments using DN and constitutively active Rac1, c-Src and Stat3 mutants are in progress to determine whether Rac1 acts downstream of, or in parallel to, the c-Src/Stat3 pathway.

Of particular importance for inhibitor design, is whether PI3K and Rac1 act cooperatively

or in parallel with c-Src/Stat3 in regulating autocrine HGF expression and Met activation in carcinoma cells. To test this possibility we will co-transfect into SP1 cells various combinations (depending on the above results) of activated or DN mutant forms of c-Src/Stat3, PI3K and Rac1, and test for HGF mRNA and protein expression, Met activation and function in our future studies.

Objective (III): To determine if up-regulating or down-regulating HGF mRNA and protein expression in nonmalignant and malignant mammary epithelial cells affects the transformed and tumorigenic phenotypes of these cells *in vitro* or *in vivo*:

***a) Effect of Upregulating HGF expression on mammary tumorigenesis and metastasis in vivo*:** During the course of this grant, other investigators have shown that constitutive expression of *HGF* in transgenic mice promotes tumor formation in many organs including breast (7-9), and that up-regulation of HGF and Met promotes neoplasia in mammary epithelial cells (50). Our results predict that upregulation of c-Src and Stat3 activity in mammary epithelial cells would enhance HGF expression and tumorigenesis. Therefore, Objective III has been revised to test this hypothesis. To test the functional significance of the c-Src/Stat3 pathway in regulating autocrine HGF loops and mammary tumorigenesis, we have initiated experiments to activate or inhibit this pathway, and to test the effect on HGF expression, autocrine activation of Met and cell function. Both mammary carcinoma cells (SP1) and nonmalignant epithelial cells (HC11) will be tested. These experiments will provide new information on the role of the c-Src/Stat3 pathway in regulating autocrine HGF loops in mammary tumorigenesis. This project is part of a new programme grant application submitted to the Canadian Breast Cancer Research Initiative.

b) Effect of downregulating HGF expression and HGF antagonism on mammary tumor progression:

i) Antisense oligonucleotides: Preliminary results to use antisense oligonulceotides to downregulate HGF expression in carcinoma cells indicated that this strategy is insufficient to significantly affect functional levels of HGF expression in carcinoma cells (data not shown). In addition, it is difficult to target this strategy preferentially to epithelial tissues. Based on work from Objectives I and II in this USAMRMC-supported project, our current focus is to target specific signalling pathways (e.g. c-Src and Stat3) that regulate HGF expression in epithelial cells (see IIIa above).

ii) HGF antagonism: It is anticipated that combined strategies may be required to inhibit HGF functions in breast tumor cells. Therefore, in addition to testing antisense oligonucleotides, we have initiated a new approach to isolate short peptide antagonists of HGF using a technique referred to as "phage display" to screen random peptide libraries (70). This approach allows the rapid identification of short peptides which bind specifically to certain growth factors or their receptors, block binding of the growth factor to its receptor, and inhibit growth factor function (71-73). These peptide "antagonists" may pave the way for a new generation of potent anti-cancer agents which can be delivered with high efficiency to the tumor site. As a first step, we have used 3-D computer modelling of the Met-binding site in the K1 kringle domain (Fig. 17), based on previously reported site-specific mutational studies and x-ray crystallography of the K1 domain (74). The results suggest the presence of a groove of approximately 7-10 a.a. in length, corresponding to the Met binding site.

Based on this information we have chosen heptapetide and decapeptide libraries for screening of libraries. HGF-binding phage will be selected from phage-display libraries using a panning procedure with recombinant NK1 peptide as absorbent. Screening of selected phage will be carried out using a high-throughput ELISA technique (70). Positive NK1-binding phage will be further characterized as possible candidates for HGF antagonism. I have recently received funds from the Canadian Breast Cancer Research Initiative to continue this study.

KEY RESEARCH ACCOMPLISHMENTS:

During this grant period, we have made the following observations:

- Elevated HGF and Met expression occurs in some newly-derived breast and non-small cell lung carcinoma cell lines.
- Several carcinoma cell lines showed autocrine tyrosine-phosphorylation of Met and increased cell survival, consistent with the presence of autocrine HGF loops in some breast and lung carcinomas.
- The ECM protein, osteopontin, is overexpressed in breast cancer, and stimulates Met activation and cell migration in the absence of exogenous HGF. This finding suggests a role of cell-substrate adhesion in regulation of HGF/Met signalling during mammary tumorigenesis.
- Activation of a c-Src/Stat3 pathway (which occurs in most breast cancers) stimulates increased expression of HGF mRNA and protein in both malignant and nonmalignant breast epithelial cells. This process may be a key step in early stage mammary tumorigenesis.
- Information from these studies could lead to novel therapeutic approaches to breast cancer.

REPORTABLE OUTCOMES:

- Published three research papers and submitted two papers for publication (see list below).
- Applied for, and received, the following grants:
  - Medical Research Council of Canada: "Role of HGF and the cell adhesion complex in survival and metastasis of carcinoma cells" C$195,000/ 3 yr (1999-2002).
  - Canadian Breast Cancer Research Initiative (IDEA): Development of peptide antagonists to HGF as a novel approach to therapeutic intervention in breast cancer C$41,500/ 2 yr (1999-2000).
- Applied for, and received, the following trainee support awards:
  - USAMRMC Postdoctoral Fellowship for Dr. Wesley Hung **#DAMD17-98-1-8330** US$80,997/ 3 yr (1998-2001): "Signal transduction in regulation of autocrine HGF expression in cancer metastasis".
  - USAMRMC Predoctoral Studentship for Jin Gui: **#DAMD17-99-1-9360** US$29,770/ 2 yr (1999-2001): "Identification of hepatocyte growth factor autocrine loops in breast carcinomas: Possible targets for therapeutic intervention".
- Applied for, and received, a sabbatical research award from the Rotschild-Yvette Mayent Research Foundation, Curie Institute, Paris, France. C$36,000 (1999-2000). "Role of c-Src and ezrin in regulation of cell-cell contacts in mammary carcinomas".
- Applied for a grant from the Canadian Breast Cancer Research Initiative for continuation of this study: "Targeting autocrine HGF loops in breast cancer metastasis" C$384,000/ 3 yr. (2001-2004). This grant is pending.

CONCLUSIONS:

We previously demonstrated elevated HGF and Met expression in regions of invasive human breast carcinomas, suggesting a role of autocrine HGF loops in invasive breast cancer. We have now demonstrated that: a) elevated HGF and Met expression occurs in some newly-derived breast and non-small cell lung carcinoma cell lines; b) several carcinoma cell lines showed autocrine tyrosine-phosphorylation of Met and increased cell survival, consistent with the presence of autocrine HGF loops; c) the ECM protein, osteopontin, stimulates Met activation and cell migration, suggesting a role of cell-substrate adhesion in regulation of HGF/Met signalling; and d) activation of a c-Src/Stat3 pathway (which occurs in most breast cancers) stimulates increased expression of HGF mRNA and protein in both malignant and nonmalignant breast epithelial cells. Thus, we have identified autocrine HGF loops which correlate with sustained cell survival in several several breast and NSCLC cell lines. We have also characterized several mechanisms that regulate autocrine HGF expression and Met activation in breast cancer; this information could lead to novel therapeutic targets. These four findings are discussed briefly below.

a) Our demonstration of HGF expression in many breast carcinoma cell lines validates our earlier observation using in situ hybridization that HGF and Met are co-expressed in carcinoma cells in regions of invasive breast cancer. Similarly, several breast carcinoma and NSCLC cell lines showed increased HGF expression. Three cell lines (SP1, SW-900 and WT-E) showed increased tyrosine phosphorylation of Met. Several cell lines showed no phosphorylation of Met although significant HGF activity was detected in conditioned media from these cell lines. Thus, HGF secreted by these cells was either insufficient or ineffective in activating Met. In one breast carcinoma cell line, MCF10A1T3B, two smaller molecular weight forms of HGF were detected, in addition to pro-HGF and mature HGF proteins, suggesting the presence of degraded native HGF, or HGF isoforms (Fig. 2). Production of naturally occurring isoforms, such as NK2 (Mr-30 kDa) which can act as an antagonist in some systems (31-33), or proteolytic degradation may have an inhibitory effect on activity of HGF produced by such carcinoma cells. Our preliminary studies (data not shown) suggest that MCF10A1T3B cells express proteases that degrade secreted HGF. Experiments involving protease inhibitors are in progress to confirm these findings.

b) Two NSCLC cell lines (SW-900 and WT-E) which express high levels of Met and secreted HGF protein, showed tyrosine-phosphorylation of Met, consistent with an autocrine HGF loop. To determine the functional relevance of the elevated Met activation in these cells, we examined DNA synthesis and cell survival. All Met-expressing NSCLC cell lines required paracrine stimulation with HGF for an optimal proliferation response, regardless of the presence of an autocrine HGF loop. In contrast, two NSCLC cell lines (SW-900 and WT-E), which express an autocrine HGF loop, showed a sustained high level of Met tyrosine-phosphorylation and cell survival under nonadherent conditions. In an independent study, Yi et al, (75) showed that NSCLC cell lines expressing HGF mRNA exhibited paracrine, but not autocrine, stimulation by HGF of DNA synthesis; however the level of constitutive activation of Met was not assessed in their study. Our results indicate that autocrine activation of Met is sufficient to stimulate cell survival, whereas additional paracrine stimulation with HGF is required to stimulate DNA synthesis in NSCLC cells. Experiments are in progress to further assess the pattern of HGF-induced signalling and functions associated with paracrine versus autocrine HGF stimulation of carcinoma cells.

c) Our finding that the ECM protein, OPN, can stimulate Met activation is novel, and supports the concept that stromal interactions are important in modulating HGF/Met expression and activity *in vivo*. Several mechanisms could be involved in regulation of Met activation by OPN including: i) OPN stimulates increased expression of HGF, leading to autocrine activation of Met; or ii) OPN facilitates ligand-independent activation of Met (76,80). We are currently examining the effect of OPN on HGF expression and activity in human mammary carcinoma cells which have been incubated with OPN or transfected with OPN cDNA. These conditions in part reflect the *in vivo* microenvironment of mammary tumors, and have been shown to be important in regulating mammary tumor progression (77-79).

d) We have demonstrated the regulation of *HGF* gene expression by c-Src kinase via Stat3 activation in breast carcinoma cells. Although many reports have indicated that increased Src kinase activity (particularly through the expression of v-Src) can stimulate gene expression via Stat3 (52-56), in this study we identify a novel target of elevated activity of c-Src kinase in breast carcinoma cells. In addition, we showed a co-operative effect of c-Src and Stat3 in the activation of *HGF* transcription in a nonmalignant mammary epithelial cell line HC11. Thus, activation of the c-Src/Stat3 signaling pathway, which occurs in most breast cancers, may override negative regulation of HGF expression and establish an autocrine HGF loop in nonmalignant epithelial cells. Our findings therefore provide an important link between breast cancer progression and HGF expression. Furthermore, the c-Src/Stat3 pathway regulating HGF expression can be a potential target for therapy in breast cancer treatment.

In summary, our results show that presence of an HGF autocrine loop is associated with a high level of cell survival in carcinoma cells, consistent with previous reports that over-expression of HGF is an indicator of poor prognosis in breast and lung cancer patients (4,6). We have also demonstrated several mechanisms that regulate autocrine HGF loops and Met activation in breast carcinoma cells. Information from these studies will provide new insights into the events involved in de-regulation of HGF in breast carcinoma cells, and may provide putative new targets, at the transcriptional or post-translational stages of HGF expression and maturation, for inhibitor designs in the treatment of breast cancer.

PUBLICATIONS FROM THIS STUDY:

1. c-SRC kinase activity is required for HGF-induced motility and anchorage-independent growth of mammary carcinoma cells. N. Rahimi, W. Hung, E. Tremblay, R. Saulnier, and B. Elliott. J. Biol. Chem. 273(50):33714-33721, 1998.

2. Co-operative effect of hepatocyte growth factor and fibronectin in anchorage-independent cell survival of mammary carcinoma cells: Requirement for phosphatidylinositol 3-kinase activity. Qiao H., Saulnier R., Patryzkat A., Rahimi N., Raptis L., Rossiter J., Tremblay E., Elliott B. Cell Growth Differ. 11:123-331999.

3. Osteopontin-induced, integrin-dependent migration of human mammary epithelial cells involves activation of the hepatocyte growth factor receptor (Met). A.B. Tuck, B.E. Elliott, C. Hotal, E. Tremblay, and A.F. Chambers. J. Cell. Biochem. 78:465-475, 2000.

4. c-Src kinase activity is required for expression of hepatocyte growth factor in breast carcinoma cells. W. Hung and B.E. Elliott. Submitted to J.Biol. Chem. Nov., 2000.

5. Identification of paracrine and possible autocrine hepatocyte growth factor loops in non-small cell lung carcinomas. Qiao, H., J. Gui, W. Hung, E. Tremblay, J. Ho, J. Klassen, B. Campling, R. Schwall and B.E. Elliott. Manuscript in preparation.

MEETING ABSTRACTS:

1. Localization of platelet-derived growth factor β-receptor, hepatocyte growth factor (HGF), and HGF receptor in nonmalignant human mammary tissue and in breast cancer. B. Elliott, J. Klassen, A. Tuck, A. Boag, B. Bhardwaj, N. Rahimi, B. Campling, S. SenGupta, and E. Sterns. DOD Era of Hope Meeting, Washington, D.C., November 2, 1997.

2. Autocrine hepatocyte growth factor-dependent signalling pathways in survival, growth and metastasis of breast carcinoma cells. B. Elliott, N. Rahimi, E. Tremblay, P. Shenoy, J. Rossiter, and R. Saulnier. DOD Era of Hope Meeting, Washington, D.C., November 2, 1997

3. Role of hepatocyte growth factor receptor (Met)-, and integrin-dependent signalling pathways in survival and growth of breast carcinoma. B. Elliott, W. Hung, H. Qiao, N. Rahimi, J. Rossiter, R. Saulnier and E. Tremblay. International Agency for Research on Cancer (IARC/WHO) Symposium on: Cell adhesion and communication in growth control and cancer. Lyon, France, Jan. 19-21, 1999.

4. A novel c-src tyrosine kinase/stat3 pathway regulates hepatocyte growth factor expression in breast carcinoma cells. W. Hung and B.E. Elliott. Meeting on "Signal transduction pathways and regulation of gene expression as therapeutic targets", DOD Era of Hope Meeting, Atlanta, Georgia, June 8-11, 2000.

5. The role of osteopontin in the malignancy of human breast carcinoma. Mechanisms of involvement in cell migration and invasiveness. A. Tuck, F. P. O'Malley, B. E. Elliott, C. Hota, E. Tremblay and A. Chambers. DOD Era of Hope Meeting, Atlanta, Georgia, June 8-11, 2000.

PERSONNEL EMPLOYED FROM THIS GRANT:

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TABLE I

Expression of HGF and Met in human breast epithelial and carcinoma cell lines

Breast Cell Line	Species	Malignant Status	HGF			Met	
			mRNA	Protein	Activity	mRNA	Protein
WO-E [a]	Human	Yes	–[f]	–	-	+	–
EL-E [a]	Human	Yes	+	+	+	+	+
HU-E [a]	Human	Yes	ND	ND	ND	ND	ND
MCF10A1[b]	Human	No	trace	+	trace	+	+
MCF10A1T3B[b]	Human	Yes	trace	+	trace	+	+
21PT[c]	Human	No	trace	ND	–	+	+
21NT[c]	Human	Yes	trace	ND	–	+	+
21MT-1[c]	Human	Yes	trace	ND	–	+	+
TM3[d]	Mouse	No	ND	–	-	ND	+
T-2410L TM6[d]	Mouse	Yes	ND	–	-	ND	+
SP1[e]	Mouse	Yes	+	+	+	+	+
SP1- 3M[e]	Mouse	Yes	+	+	+	+	+

Table I Legend:

a) EL-E, WO-E and HU-E are human breast carcinoma cell lines derived from human breast cancer patients (obtained from Dr. B. Campling, Cancer Research Lab., Queen's University).

b) MCF10A1 is a subclone of a spontaneously immortalized non-tumorigenic human breast epithelial cell line established from long term culture of a breast subcutaneous mastectomy. MCF10A1T3B is a cell line derived from *Ha-Ras* transfected MCF10A1 cells growing as a tumor in a nude mouse (obtained from Dr. F. Miller, Michigan Cancer Foundation).

c) Cell lines were derived from a patient with infiltrating breast carcinoma (obtained from Dr. R. Sager, Dana Farber Cancer Institute).

d) TM3 is a Balb/c mouse-derived mammary epithelial cell line (obtained from D. Medina, Baylor College). T-2410LTM6 is a carcinoma cell line derived from TM3 cell.

e) SP1 is a murine mammary carcinoma which arose spontaneously in a CBA female mouse. SP1-3M is a highly metastatic variant subclone of SP1 selected by serial passage of a metastatic nodule into the mammary fat pad.

f) ND, not determined; +, positive; -, negative.

TABLE II

Expression of HGF and Met in human Non-small cell lung carcinoma cell lines

Carcinoma Cell Lines	Histology	Origin	HGF			Met		HGF-induced DNA synthesis[c]
			mRNA	Protein	Activity	mRNA	Protein	
SW-900	squamous cell carcinoma	primary tumor	+[b]	+	+	+	+	+
WT-E	squamous cell carcinoma	pleural effusion	trace	+	-	+	+	+
SK-Luci-6	large cell anaplastic	primary tumor	+	+	+	+	-	-
QU-DB	large cell anaplastic	primary tumor	trace	-	-	+	-	-
BH-E	adenocarcinoma	pleural effusion	+	+	+	+	-	-
LC-T	adenocarcinoma	primary tumor	-	-	-	+	-	-

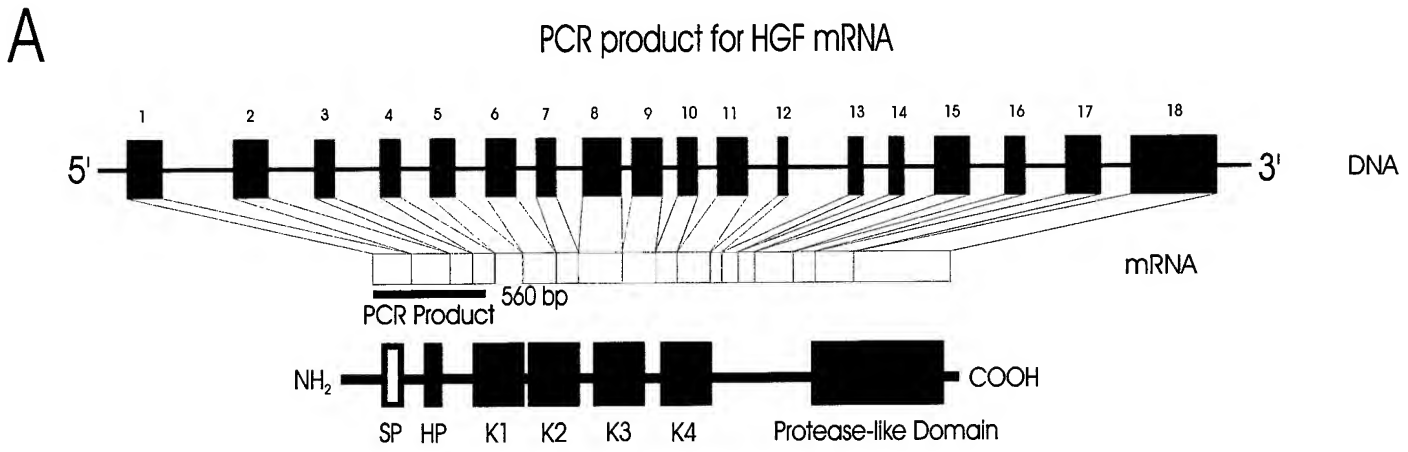
Legend:

a) See Materials and Methods for designation of Non-small cell lung carcinoma cell lines.

b) +, positive; -, negative.

c) Cells ($1x10^4$) in triplicate were incubated in a 24 well plate for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 h, ^{3}H-Thymidine was added, and cells were incubated for a second 24 h period. DNA synthesis was measured as incorporation of ^{3}H-Thymidine.

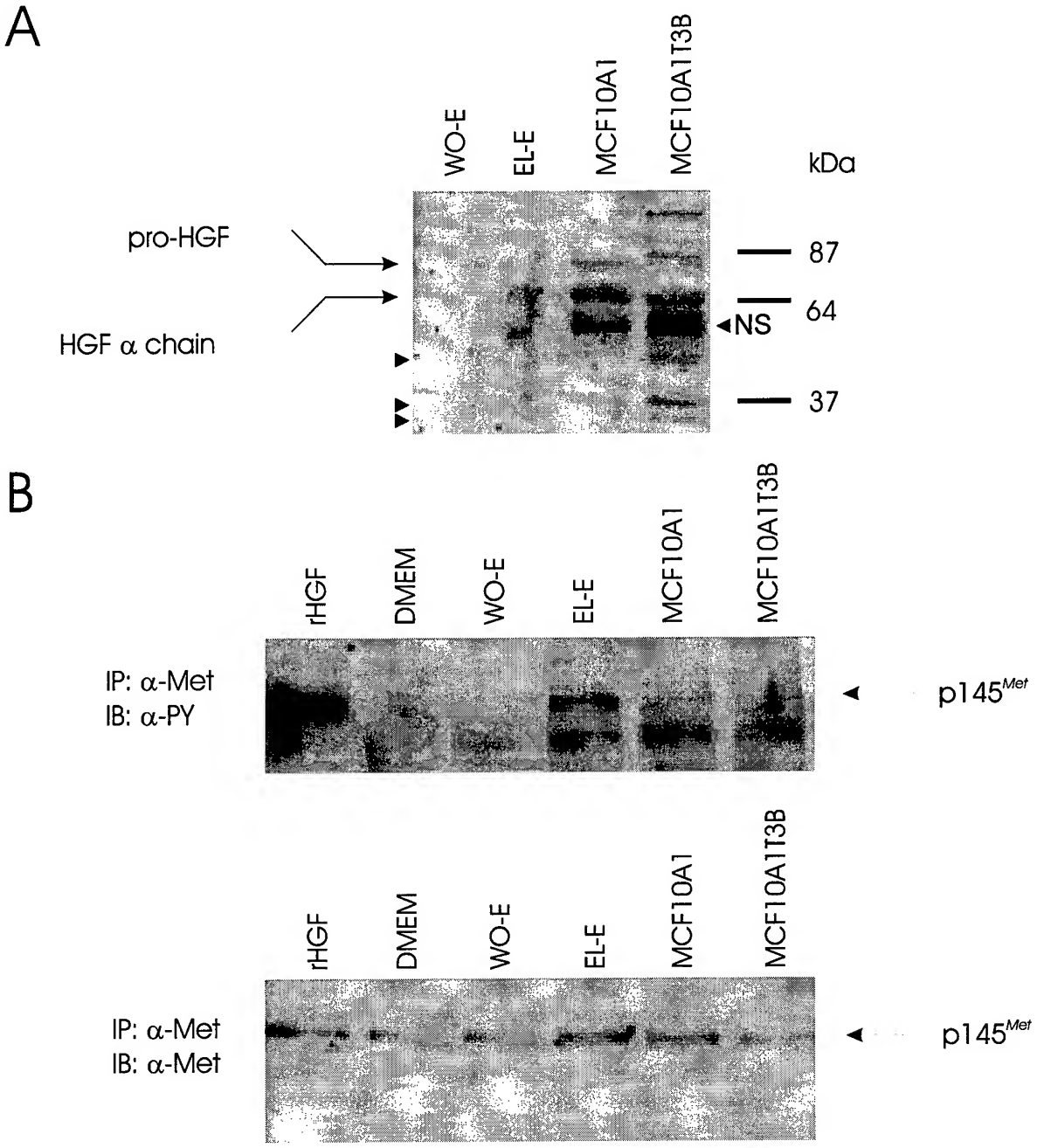
Figure 1



Design of PCR primers for detection of HGF mRNA

Panel A) Primers were designed to overlap more than one exon and to crossreact between mouse and human HGF primers: 5' (sense) TGT CGC CAT CCC CTA TGC AG (corresp. to bases 69-88 of hHGF); 3' (antisense) TCA ACT TCT GAA CAC TGA GG. (corresp. to bases 610-629 of hHGF). Panel B) cDNA was prepared from 1 µg of total RNA, and subjected to RT-PCR of 25 cycles of: 1 min at 95°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (elongation). Lane 1: DNA molecular size markers; lane 2, 206 bp marker; lane 3, PCR product of HGF.

Figure 2

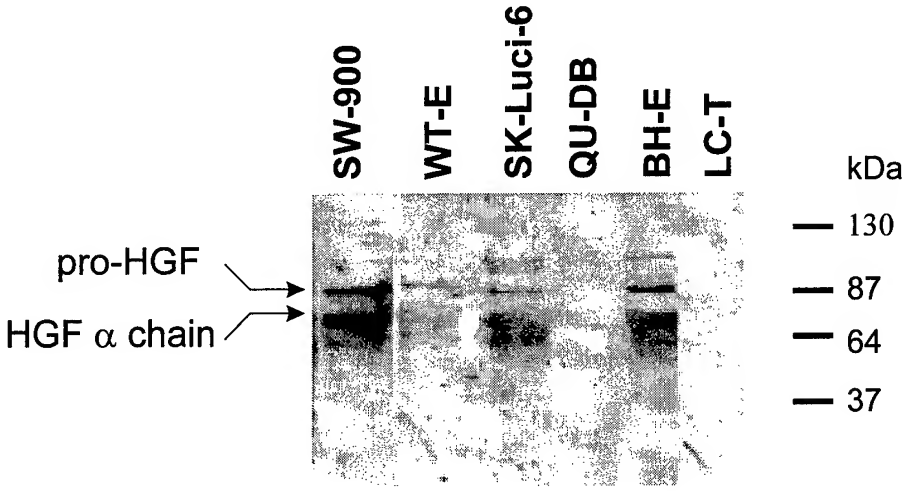


Expression and activity of HGF secreted by human breast carcinoma cell lines

Panel A) Conditioned media (CM) from newly established human breast carcinoma cell lines were subjected to Cu(II)-affinity chromatography (42), and eluted proteins were subjected to SDS-PAGE under reducing conditions. Western blotting with anti-HGF IgG revealed bands corresponding to pro-HGF and mature HGF α-chain. Arrows indicates putative degradation fragments and/or isoforms of HGF. A non-specific band (NS) at 55 kDa was present in most lanes. Panel B) CMs from cell lines in A) were tested for HGF activity as determined by the ability to stimulate tyrosine-phosphorylation of Met in A549 cells, which express Met, but not HGF. CM from EL-E showed stronger activity of HGF, than from MCF10A1T3B. See Table I.

Figure 3

A

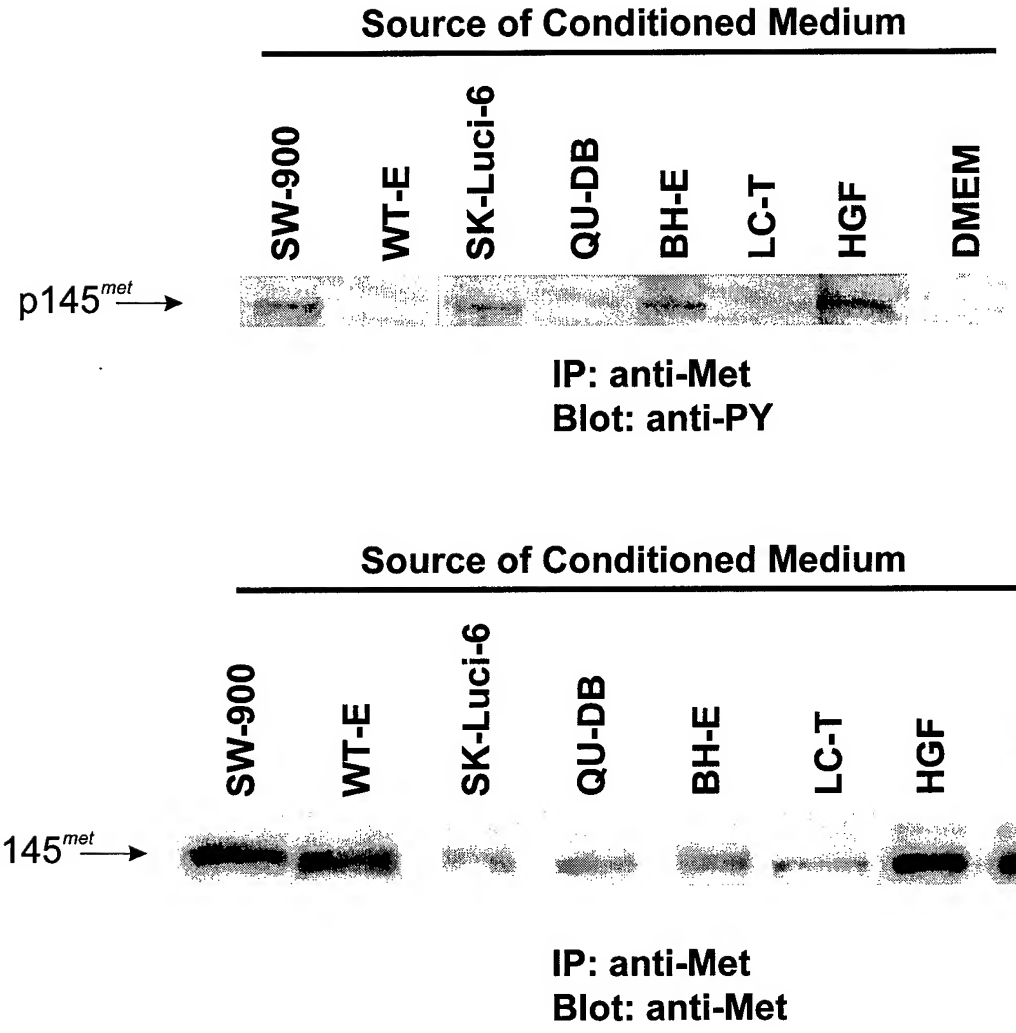


Detection of HGF protein in conditioned media of NSCLC cell lines

The presence of HGF protein in the conditioned media collected from different lung carcinoma cell lines was determined using copper (II) affinity chromatography to purify putative HGF from conditioned media. Fractions containing putative HGF were concentrated by Microcon concentrators, and were analysed by reducing SDS-PAGE, followed by western blotting with sheep anti-HGF antibody (Genentech). Immunoreactive bands were revealed by ECL. Arrows corresponding to pro-HGF and mature HGF are shown.

Figure 3

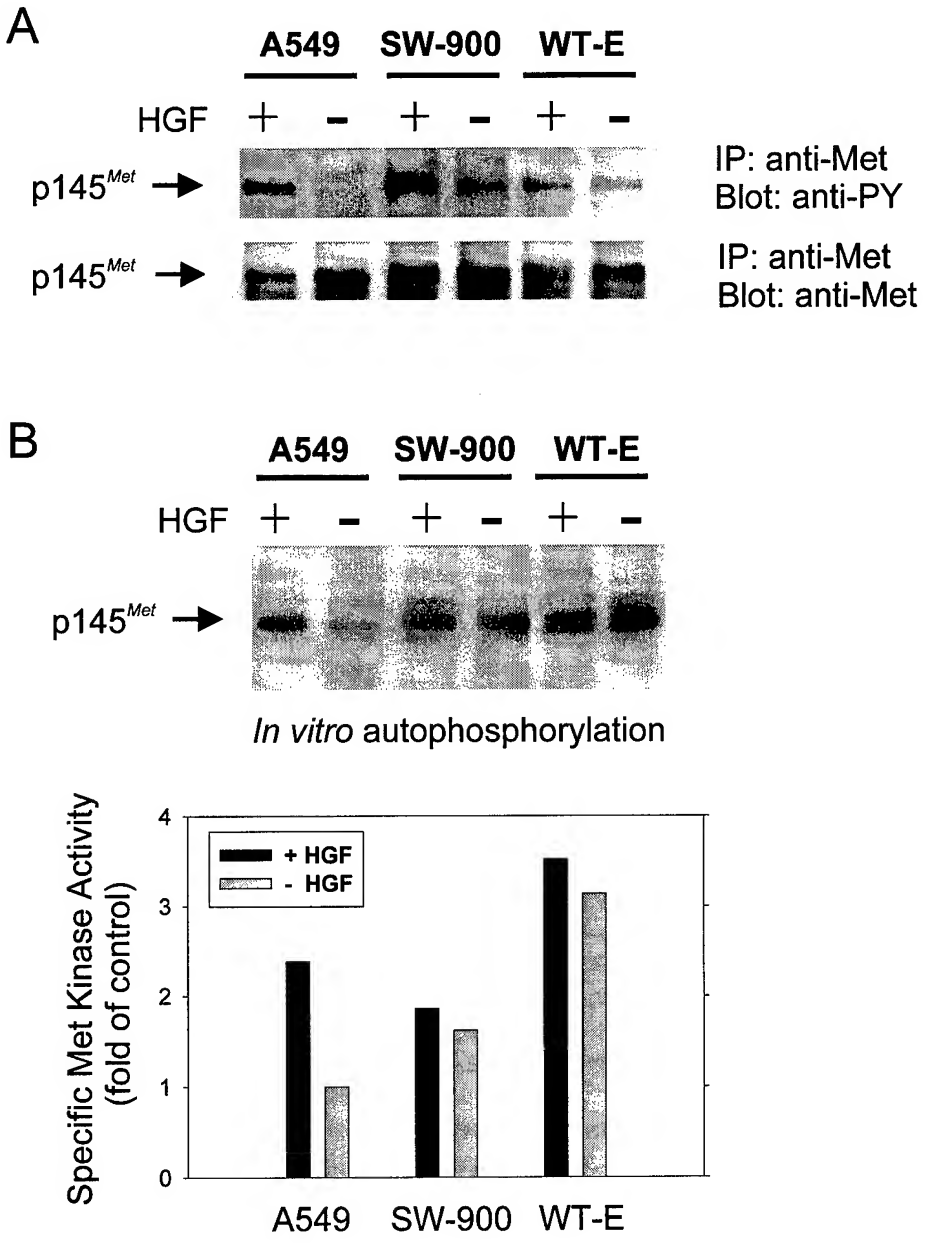
B



HGF activity in conditioned media from NSCLC cell lines

A549 cells which express Met, but not HGF, were prestarved before incubation with conditioned media from various cell lines. Controls consisted of cells incubated without, or with, HGF (40 ng/ml). After 30 min of incubation at 37°C, cells were washed with ice-cold PBS, lysed in lysis buffer, and immunoprecipitated with rabbit anti-Met antibody. Immunoprecipitates were washed several times with lysis buffer before being analysed by reducing SDS-PAGE. Proteins were analyzed using western blotting with anti-phosphotyrosine antibody (anti-PY) (upper) or with anti-Met antibody (lower).

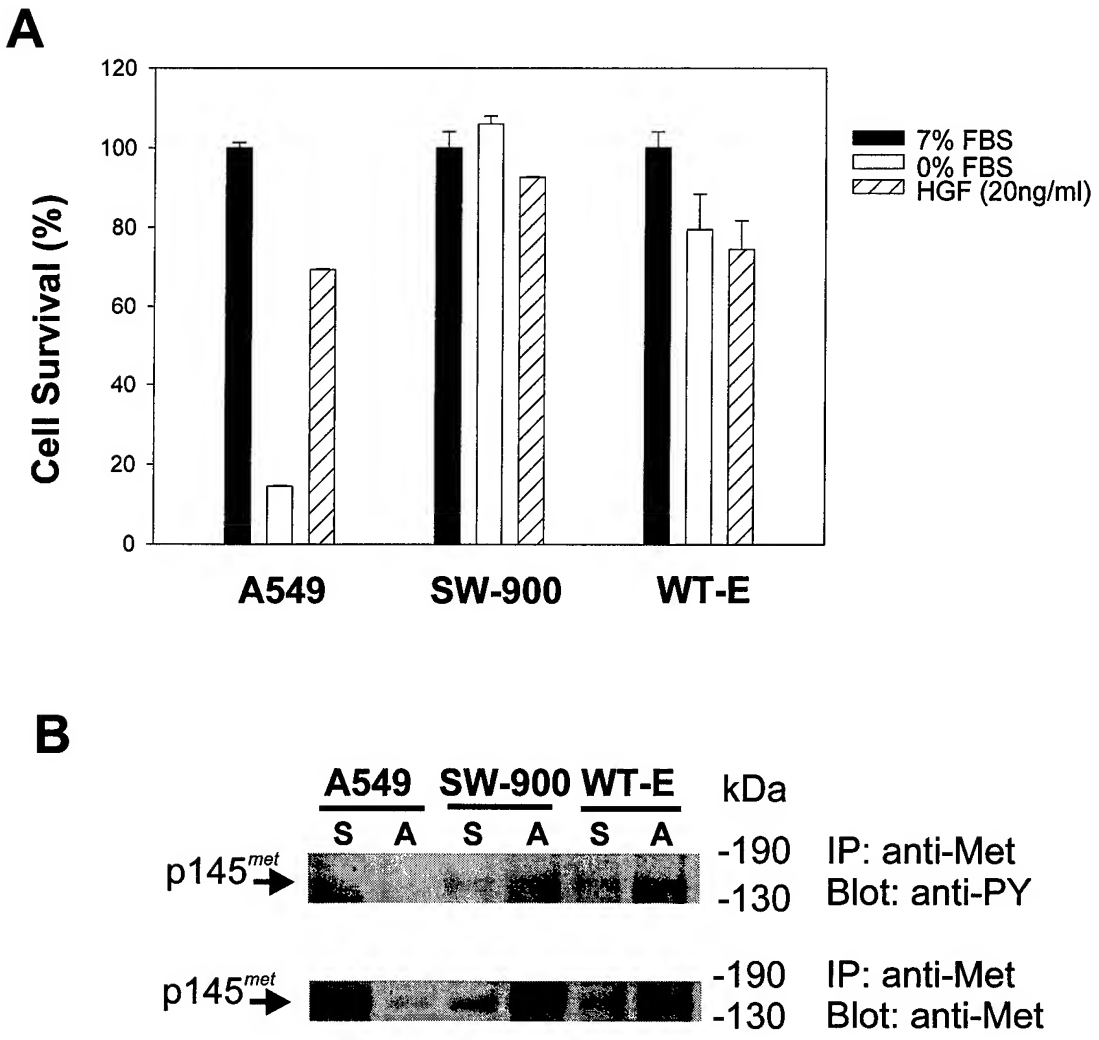
Figure 4



Met is constitutively active in SW-900 and WT-E cells

A549, SW-900 and WT-E NSCLC cell lines were cultured to 80% confluence and serum-starved overnight. The cells were then treated with HGF (20 ng/ml) for 20 min at 37°C and lysed. Clarified cell extracts were normalized for protein concentration and precipitated with anti-Met IgG. Panel A: Half of the immunoprecipitates were analysed by western blotting. The blot was probed with anti-PY antibody, and the bands were visualized with ECL reagents. The same blot was stripped and re-probed with anti-Met IgG as a loading control. Panel B: Half of the immunoprecipates were assayed for Met kinase activity *in vitro* (upper) (See App. II). The signal densities were measured with a PhosphoImager and plotted relative to the control A549 cells without HGF (lower).

Figure 5

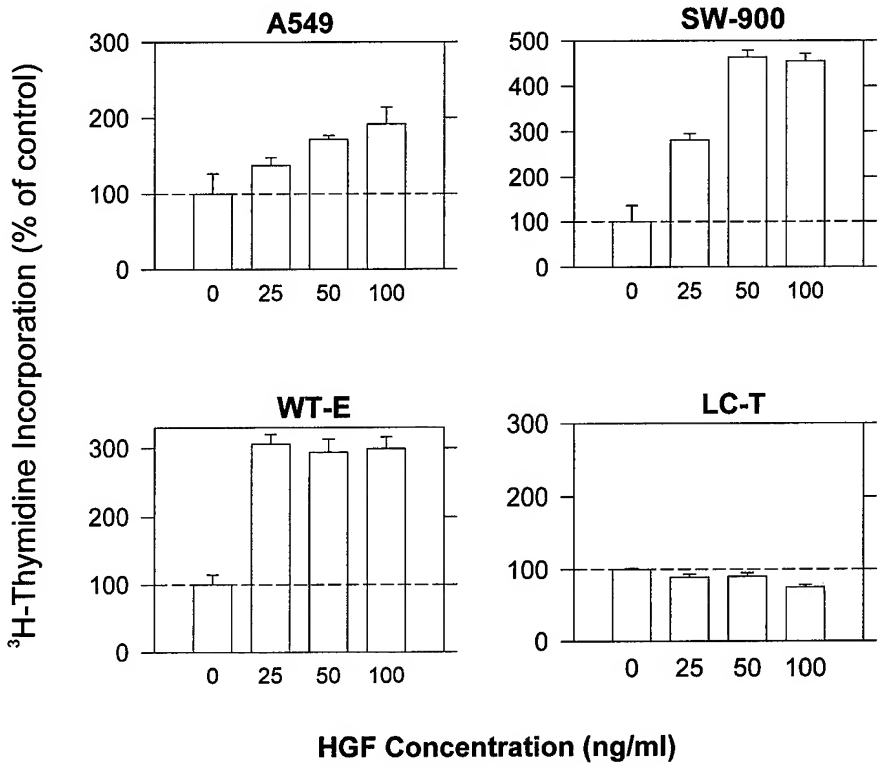


SW-900 and WT-E cells show a high level of survival under anchorage-independent conditions

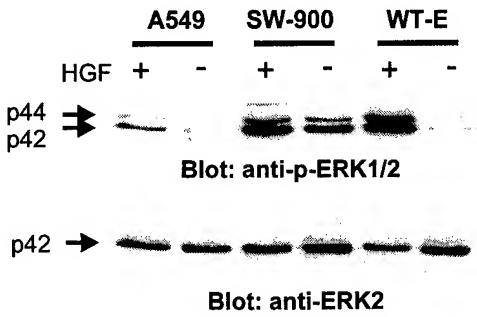
Panel A: A549, SW-900 and WT-E cells were serum-starved overnight, and seeded in suspension cultures with the treatments as indicated. After 24 h incubation at 37°C, cells were transferred to a 96 well plate and surviving cells were measured with an MTS colorimetric assay. The results are expressed as mean ± range of duplicates, and are representative of two experiments. Panel B: The cells were serum-starved and put in suspension (S) or left on plates (A) for 4 h in 37°C. The cells were then lysed and cell extracts were immunoprecipitated with anti-Met IgG and analyzed by western blotting with anti-PY or anti-Met antibodies.

Figure 5

C



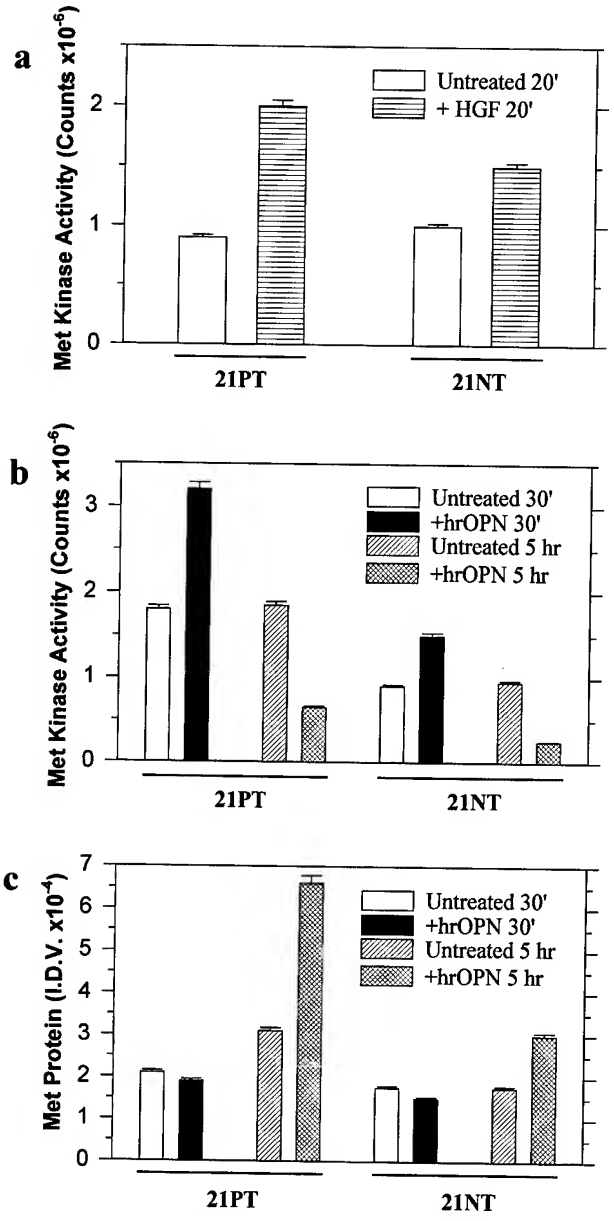
D



Exogenous HGF stimulates DNA synthesis and phosphorylation of ERK 1/2 in A549, SW-900 and WT-E cell lines

Panel C: Cells were serum-starved overnight and then treated without, or with, HGF at the concentrations indicated. A control consisted of the LC-T cell line (HGF and Met negative). After 24 h, 0.2 μCi of [^{3}H]thymidine was added, and cells were incubated for an additional 24 h. Cells were then harvested, transferred to filters, and the incorporation of [^{3}H]thymidine was measured using a scintillation counter. Results are expressed as the mean cpm of quadruplicate wells ± S.D. Panel D: The cells were serum-starved overnight and treated without,or with, HGF (20 ng/ml) for 20 min and then lysed. The cell extracts were analyzed by western blotting with anti-phospho-ERK1/2 antibody. The same blot was stripped and re-probed with anti-ERK2 antibody to confirm equal protein loading between groups.

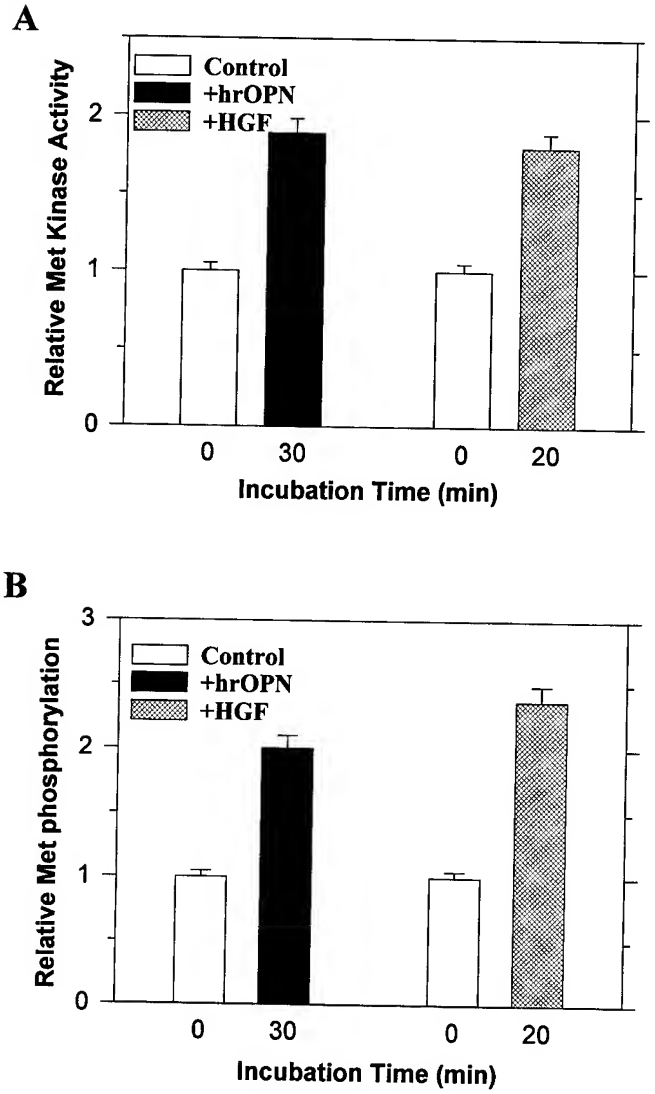
Figure 6



HGF (panel A) and OPN (panel B) -induced increase in total cellular Met kinase activity of 21PT and 21NT cells. OPN (panel C) -induced increase in total Met protein of 21PT and 21 NT cells

Panel A) and Panel B) Cells were incubated +/- 20 ng/ml HGF or 50 µg/ml hrOPN for the times indicated, and cell lysates were prepared. Equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG, and *in vitro* Met kinase activity was determined as described in App. III. Quantitation was done using a Phosphoimager. Total Met kinase activity is expressed in cpm/sample. Panel C) Met protein was quantitated by immunoprecipitation with rabbit polyclonal anti-Met antibody, followed by 7% SDS-PAGE and western blotting. Total Met protein was quantitated by densitometry and is expressed in integrated density value units.

Figure 7



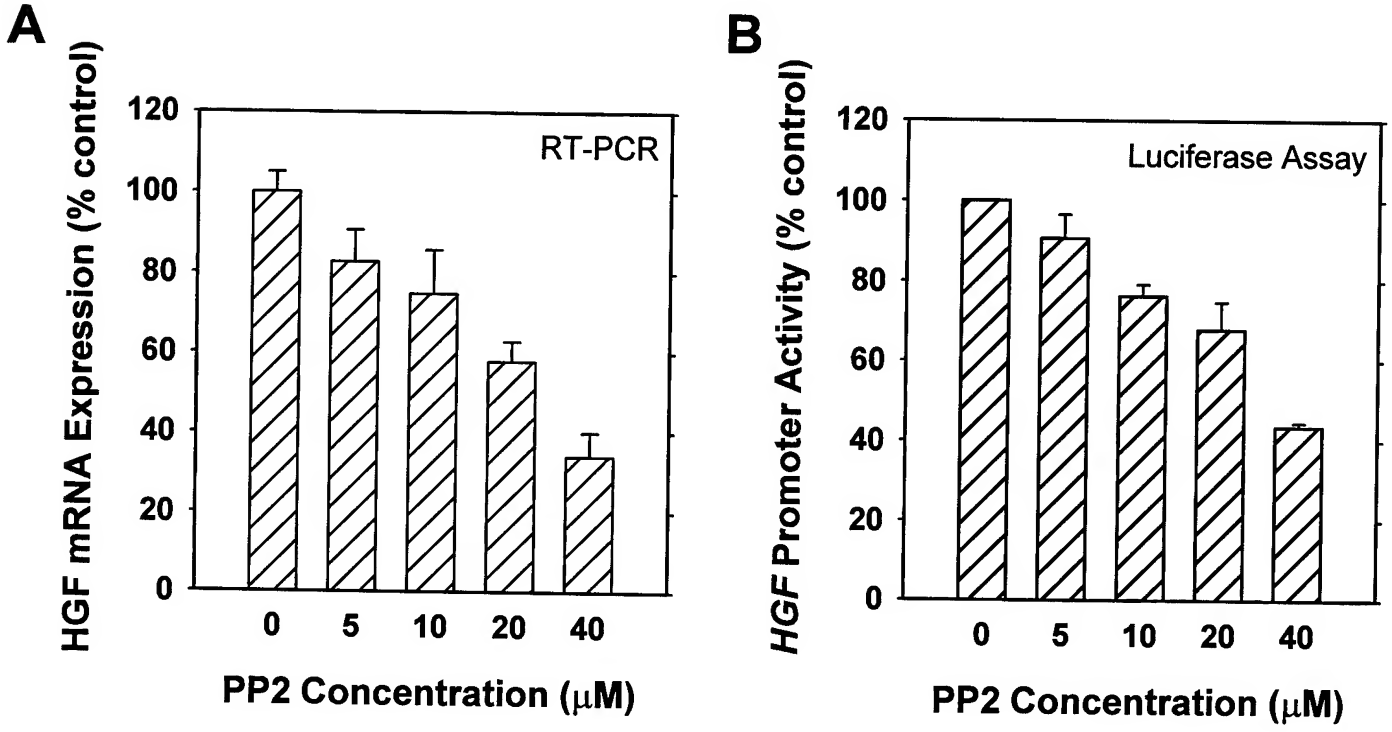
Induction of specific Met kinase activity (panel A) and Met tyrosine phosphorylation (panel B) of MDA-MB-435 cells by HGF and OPN

MDA-MB-435 cells were incubated +/- 50 µg/ml hrOPN or 20 ng/ml HGF for the times indicated. Cell lysates were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG. As levels of total Met protein were higher and fluctuated more in MDA-MB-435 cells than in 21PT and 21NT, activation of Met protein in MDA-MB-435 was more appropriately expressed as Relative Met kinase activity (A) and tyrosine phosphorylation (B).

Panel A) *In vitro* Met kinase activity was assayed as described in Materials and Methods. Relative Met kinase activity, normalized to total Met protein, was quantitated using a Phosphorimager.

Panel B) Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with 1% BSA in TBST, and probed with anti-mouse antibody and ECL. Relative Met tyrosine-phosphorylation normalized to total Met protein was quantitated by densitometry.

Figure 8

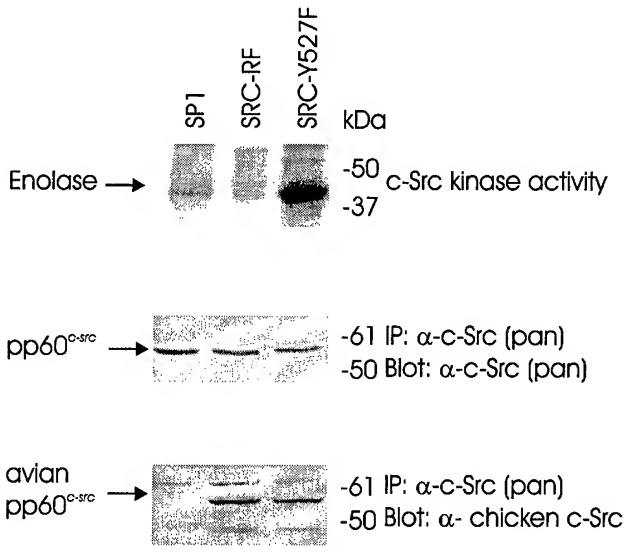


Treatment with the c-Src family kinase inhibitor PP2 decreases HGF mRNA level and transcription

Panel A: Prestarved SP1 cells were incubated with the Src family kinase inhibitor PP2 at the concentrations indicated. After 24 hours, cells were lysed and total RNA was extracted. The amount of *HGF* mRNA in each sample was quantitated using RT-PCR with HGF-specific primers and primers for GUS B (see App. III). The amount of *HGF* mRNA was normalized to GUS B mRNA and the level of *HGF* mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments +/- range.

Panel B: SP1 cells were transfected with a reporter plasmid containing the 2.7 kb fragment of the *HGF* promoter driving expression of the luciferase gene (2.7 HGF-luc). A β-galactosidase expression plasmid was co-transfected in each group for normalization to account for differences in transfection efficiency. After 24 h of incubation, PP2 was added at the concentrations indicated, and the cells were incubated for an additional 24 h, lysed and assayed for luciferase activity. Luciferase activity of each sample was expressed as percentage of untreated (control) cells. Values represent the mean +/- SD of triplicate samples. The experiment was done twice with similar results.

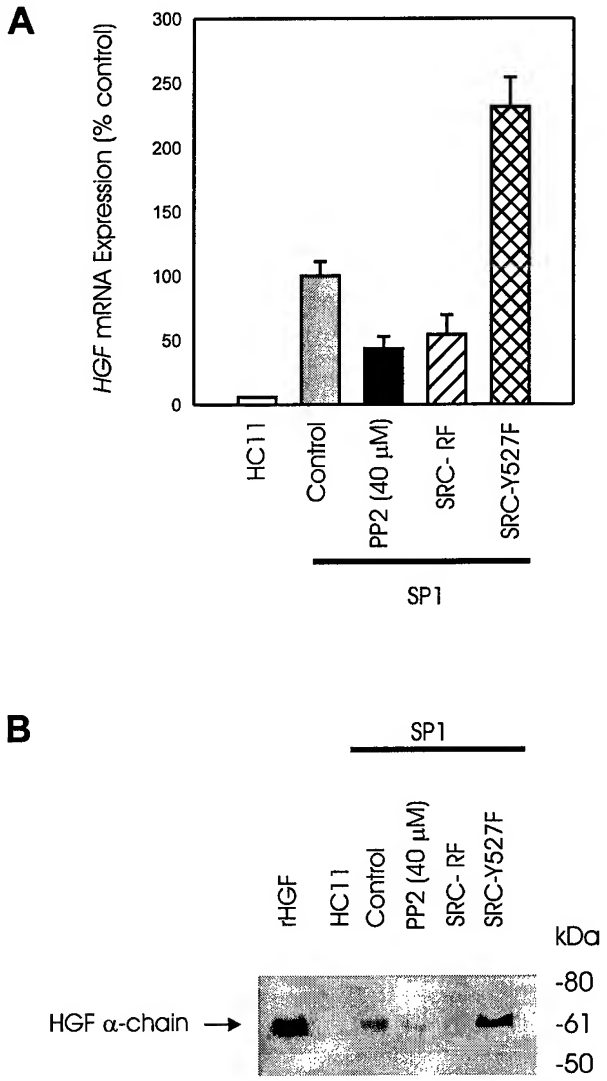
Figure 9



Ectopic expression of c-Src kinase mutants in SP1 cells

SP1 cells were transfected with expression vectors containing activated c-Src (SRC-Y527F) or dominant negative c-Src (SRC-RF) or an empty expression vector (SP1). After 48 hours, cells were lysed. Equal amounts of the lysates were immunoprecipitated with anti-c-Src antibody. Half of the immunoprecipitates was used to detect c-Src kinase activity using enolase as a substrate (upper). The other half was subjected to western blotting with anti-Src antibody to determine total c-Src protein content in the immunoprecipitates (middle), and then reprobed with monoclonal anti-chicken c-Src (EC10) antibody to detect the level of ectopic expression of each c-Src mutant(lower).

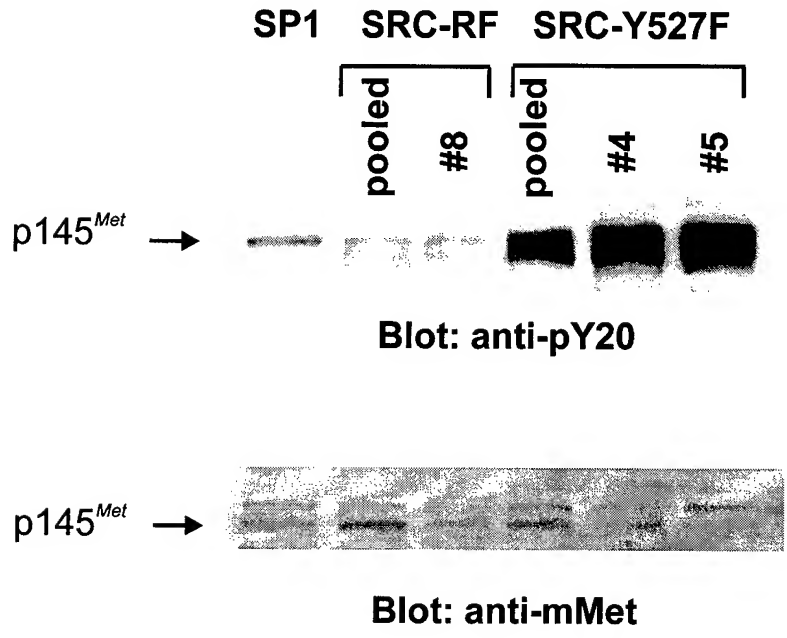
Figure 10



c-Src kinase activity modulates *HGF* mRNA and protein levels in SP1 cells

Panel A: SP1 cells transfected with dominant negative Src (SRC-RF) or activated Src (SRC-Y527F) or empty vector (control) were prestarved overnight. PP2 (40 μM) was added to one plate of SP1 cells and incubated for an additional 24 h. A nonmalignant breast epithelial cell line HC11 was used as a negative control. Total RNA was isolated, and the amount of *HGF* mRNA in each sample was quantitated using RT-PCR and normalized to GUS B mRNA as described in Fig. 1. The level of *HGF* mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments +/- range. Panel B: Serum-free conditioned media were collected for 24 h from HC11 cells, PP2-treated SP1 cells, and SP1 cells transfected as in Panel A. HGF protein from the conditioned media was purified using copper (II) affinity chromatography (42). The fraction containing HGF protein was concentrated in Microcon concentrators and subjected to denaturing SDS-PAGE. Recombinant HGF (100 ng) was included in one lane as a control. After electrophoresis, the proteins were transferred onto nitrocellulose and the blot was probed with anti-HGF antibody. Immunoreactive bands were revealed using Enhanced Chemiluminescence kit.

Figure 11

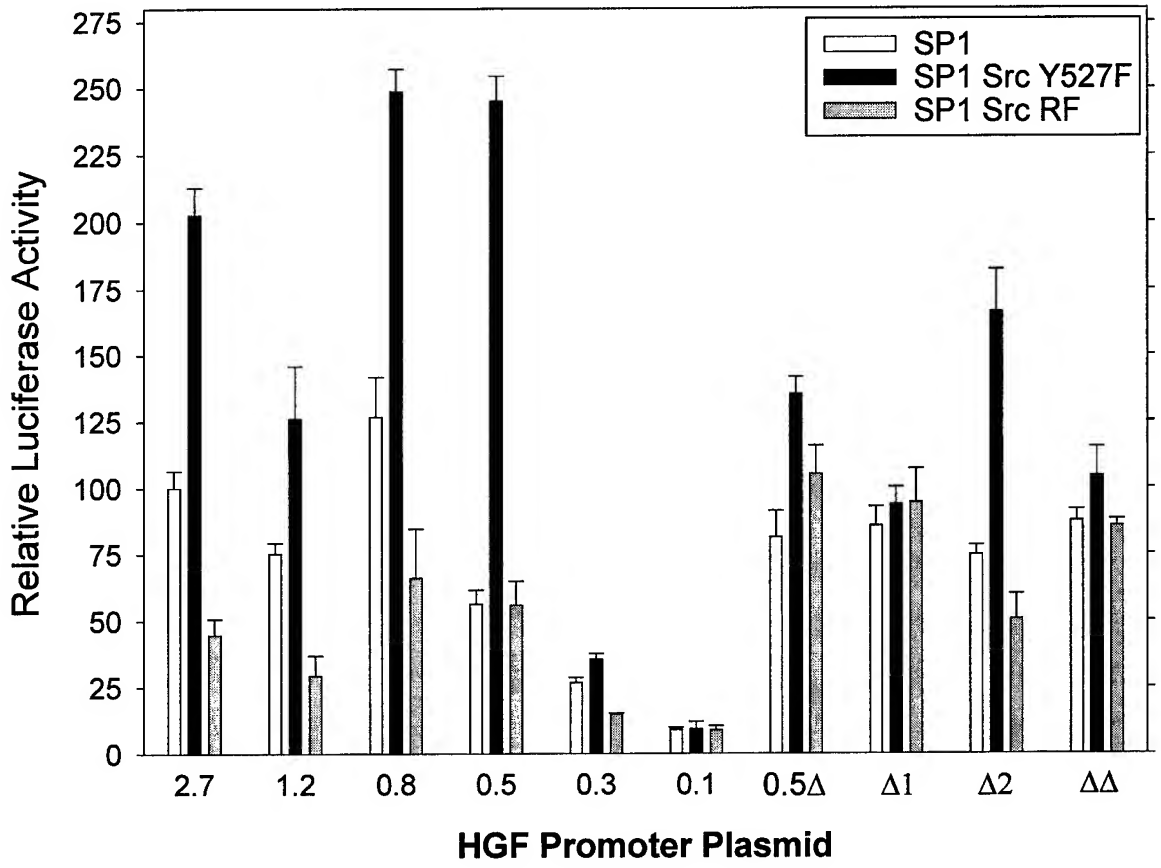


c-Src Kinase promotes Met activation in SP1 cells

SP1 cells transfected with activated (SRC-Y527F) or dominant negative (SRC-RF) mutants of c-Src were serum-starved overnight and re-plated on fibronectin substratum for 30 min at 37°C. Cell lysates were subjected to 8% reducing SDS-PAGE, and analyzed by western blotting with anti-PY or anti-Met antibodies. SRC-Y527F cells (pooled and sub-cloned) showed a significant increase in tyrosine-phosphorylation of Met, compared to SRC-RF cells.

Figure 12

A

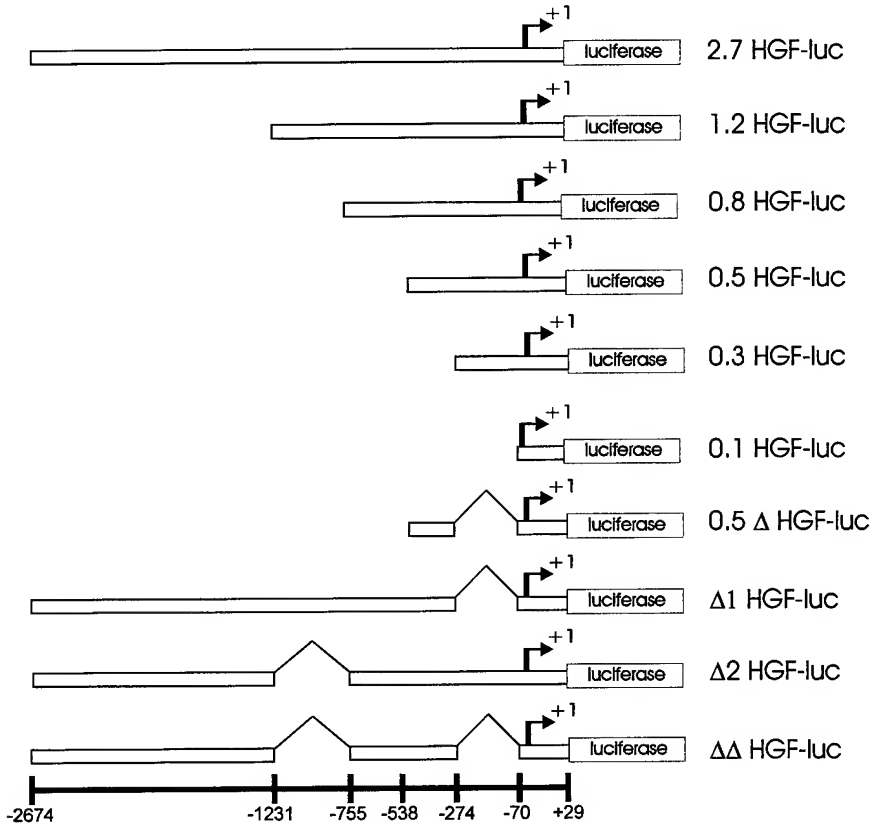


c-Src kinase responsiveness of *HGF* transcription requires the -254 to -70 bp region of the *HGF* promoter

Panel A: SP1 cells were co-transfected with the *HGF*-luciferase reporter (2.7 HGF-luc), reporter constructs containing various deletions of the *HGF* promoter (see Panel B), and activated c-Src (SRC-Y527F), dominant negative c-Src (SRC-RF) or an empty expression vector (control). Luciferase activity was determined, and normalized in each group as described in Fig. 8B. Values represent mean +/- SD of triplicate samples. The experiments were done three times using two different preparations of plasmid DNA with similar results.

Figure 12

B

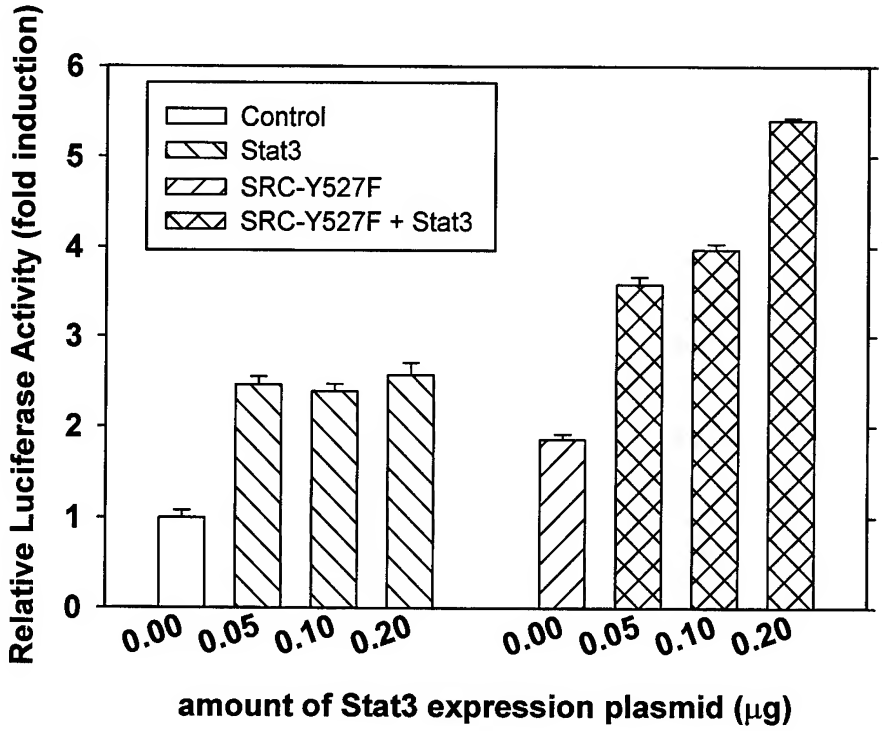


c-Src kinase responsiveness of *HGF* transcription requires the -254 to -70 bp region of the *HGF* promoter

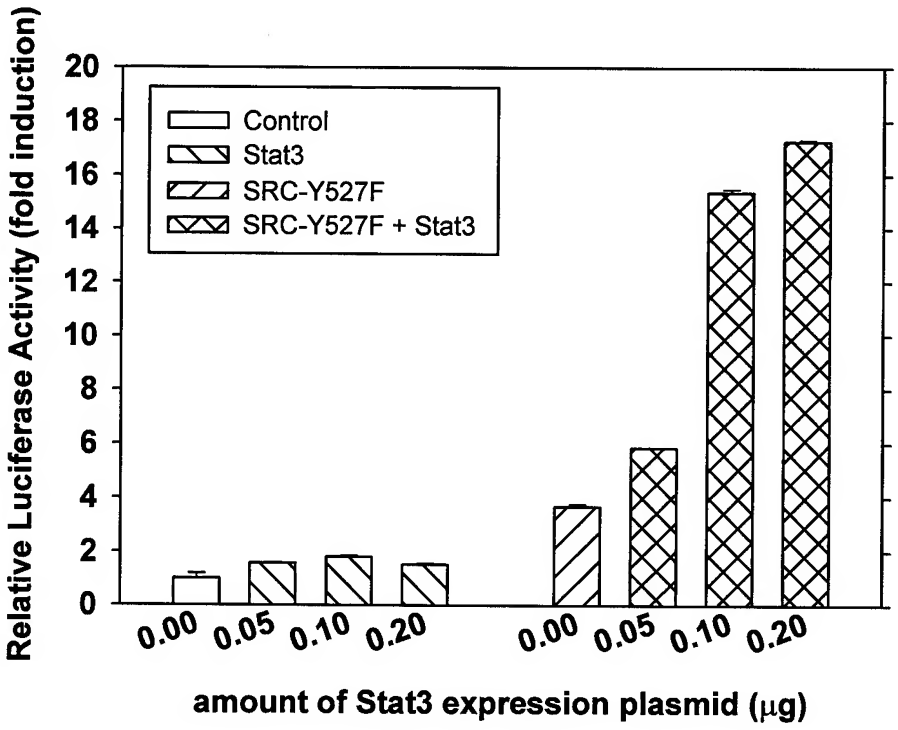
Panel B: Schematic representation of the wildtype *HGF* reporter construct and the corresponding internal deletion mutants used in Panel A is shown. The name of each construct refers to the full length (2.7 kb) or truncated promoter sequences (1.2, 0.8, 0.5, 0.3, 0.1 kb) upstream of the transcriptional start site (indicated by arrow). In addition, constructs containing a 0.5 kb sequence with an internal deletion of the region between –254 and –70 (0.5Δ), or the full length sequence containing internal deletion of regions between –254 and –70 (Δ1), -1231 and –755 (Δ2), or both (ΔΔ) were used.

Figure 13

A. SP1 cells



B. HC11 cells

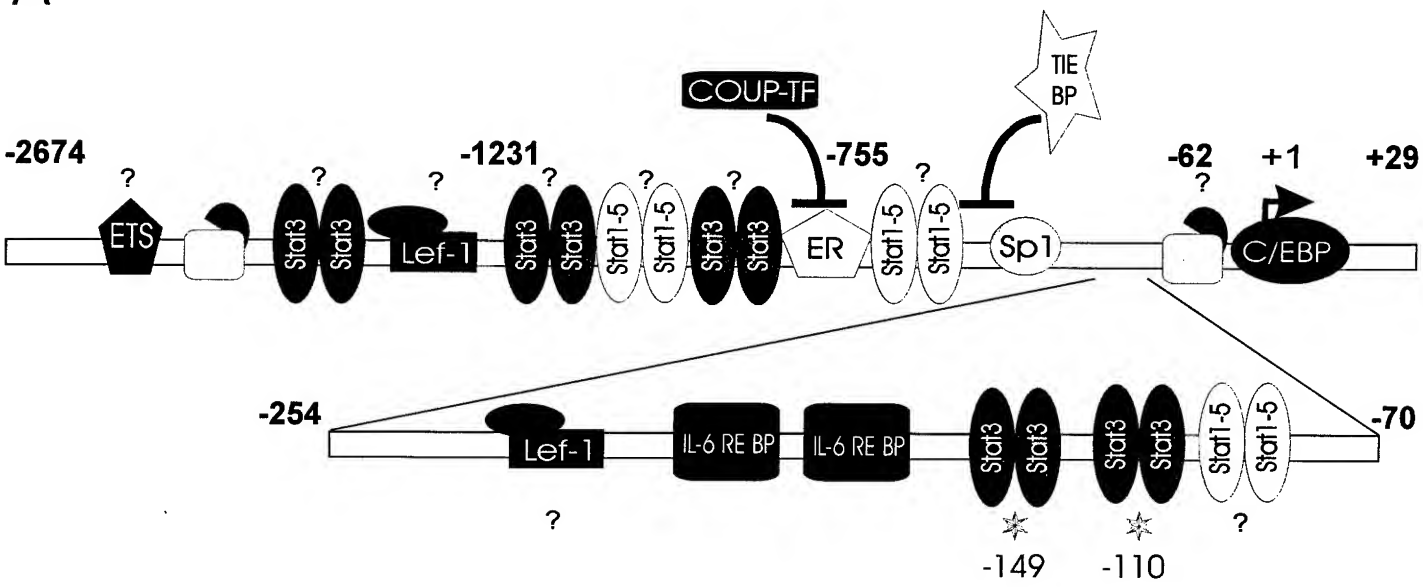


Stat3 induces HGF transcription in co-operation with activated c-Src

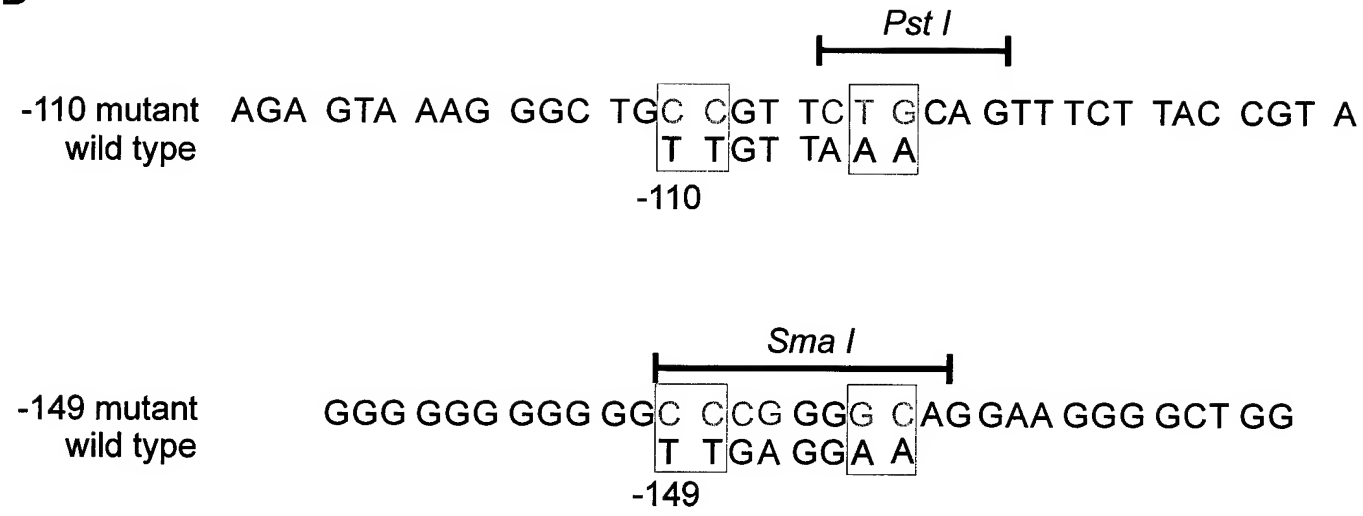
SP1 carcinoma cells (Panel A) and HC11 mammary epithelial cells (Panel B) were co-transfected with the 2.7 HGF-luc reporter and activated c-Src (SRC-Y527F) or an empty vector (control), in combination with varying amounts of Stat3. Luciferase activity was determined and expressed as a percentage of that in control cells as described in Fig. 8B. Values represent the mean +/- SD of triplicate samples. The experiments were done twice with similar results.

Figure 14

A



B

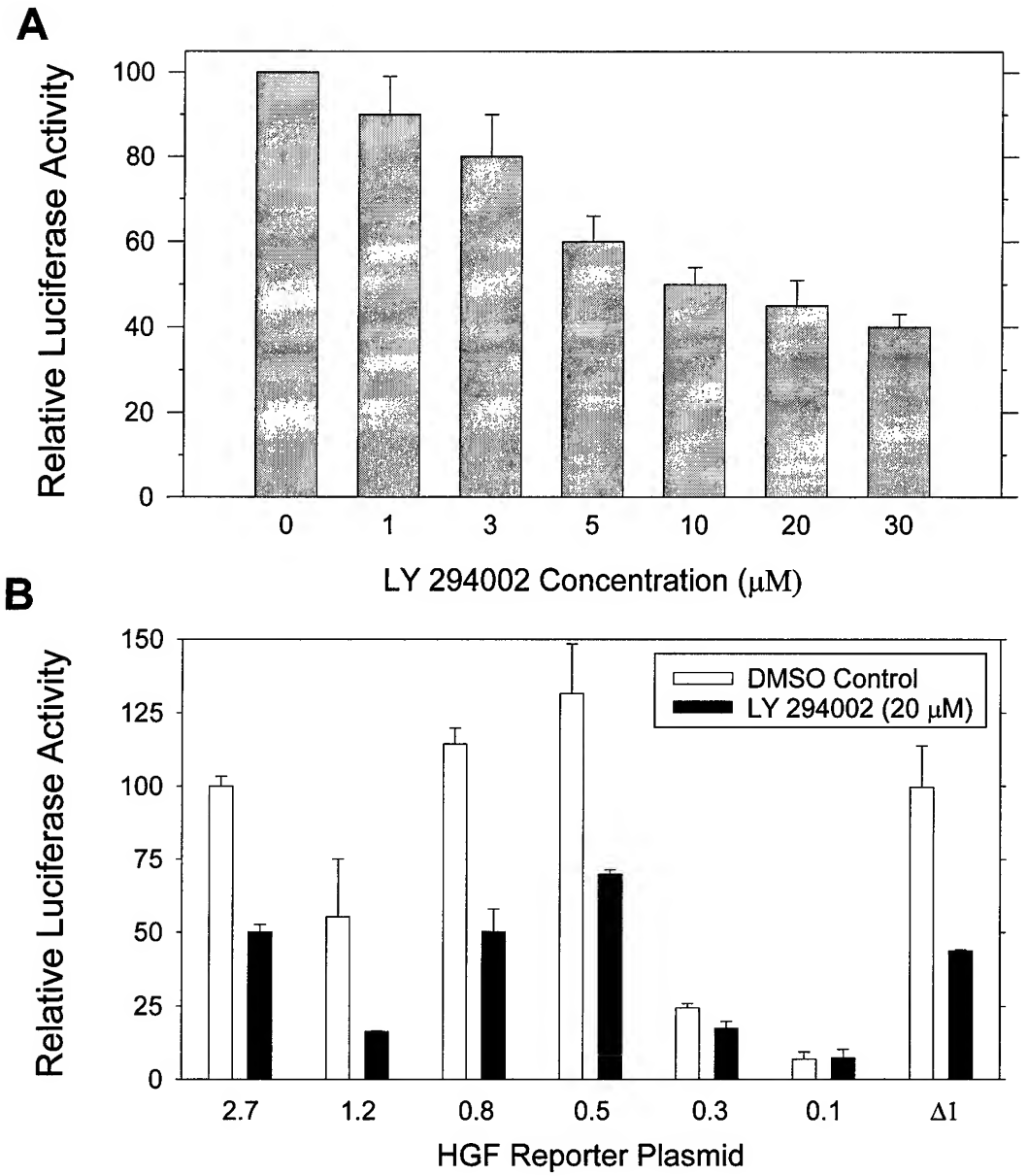


Proposed organization of *HGF* promoter and primers for mutagenesis

Panel A) A proposed organization of the HGF promoter is shown. Putative consensus sites are indicated by "?". The c-Src responsive region is enlarged, and the putative Stat3 consensus sites proposed in this study are indicated by"*".

Panel B) The design of primer sequences to be used for mutagenesis for the -110 and the -149 sites, respectively, is shown. The mutated bases are indicated in red. Restriction sites marking each mutation are also shown.

Figure 15



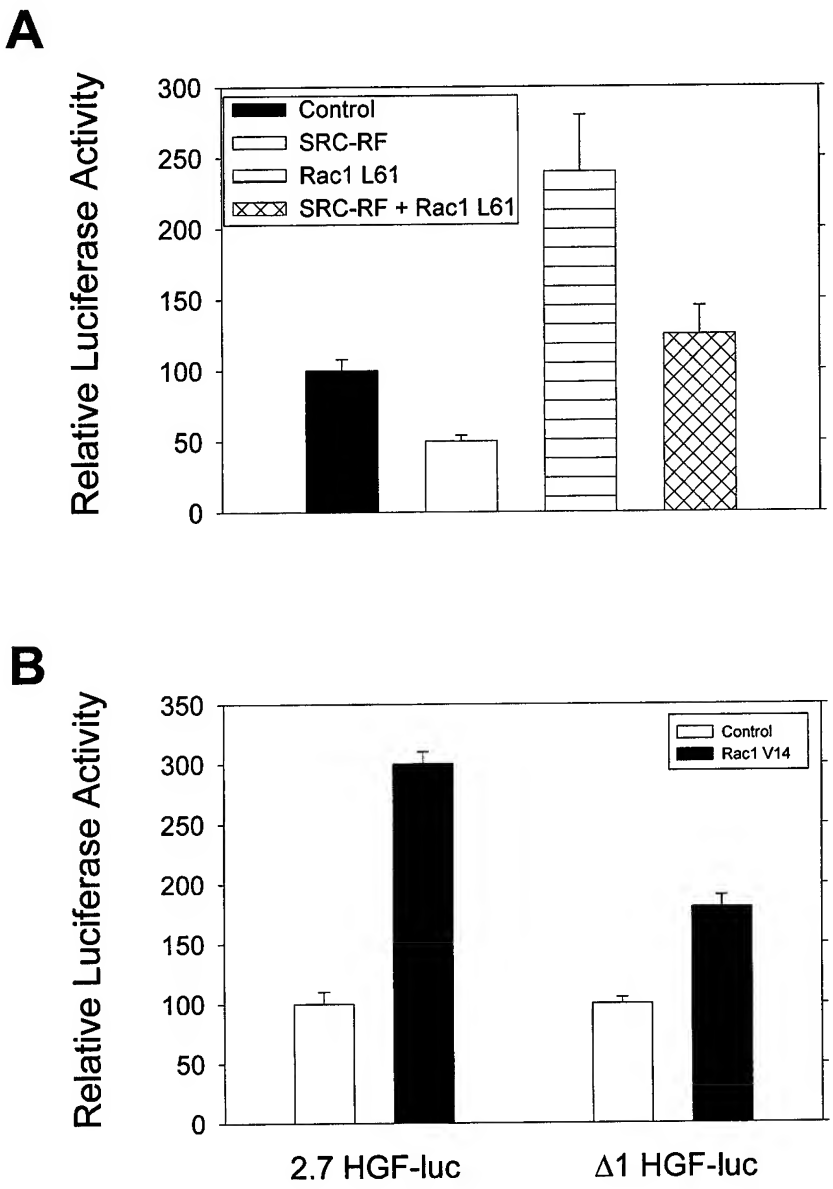
The PI3K inhibitor LY294002 blocks HGF promoter activity in SP1 cells

Panel A: SP1 cells were seeded overnight in a 24 well plate and co-transfected with a full length HGF promoter reporter plasmid (2.7 *HGF*-luc) and a β-galactosidase expression vector (pSG5-βgal). After 24 h, cells were treated with the indicated concentrations of LY294002 and incubated for an additional 24 hrs. Cells were lysed; luciferase and β-galactosidase activities were determined, and mean relative luciferase activity was normalized to control (untreated) cells. Bars indicate S.D. of triplicates.

Panel B: SP1 cells were co-transfected with the indicated *HGF*-luc reporter plasmids (Fig. 12B) and pSG5-βgal as described in Panel A. Results are expressed as mean relative luciferase activity normalized to the 2.7 *HGF*-luc level in untreated SP1 cells.

LY294002 inhibition of HGF promoter activity was unaffected by deletions from -2.7 kb to -0.5 kb, nor by the Δ1 HGF deletion mutant (lacking the c-Src responsive region).

Figure 16



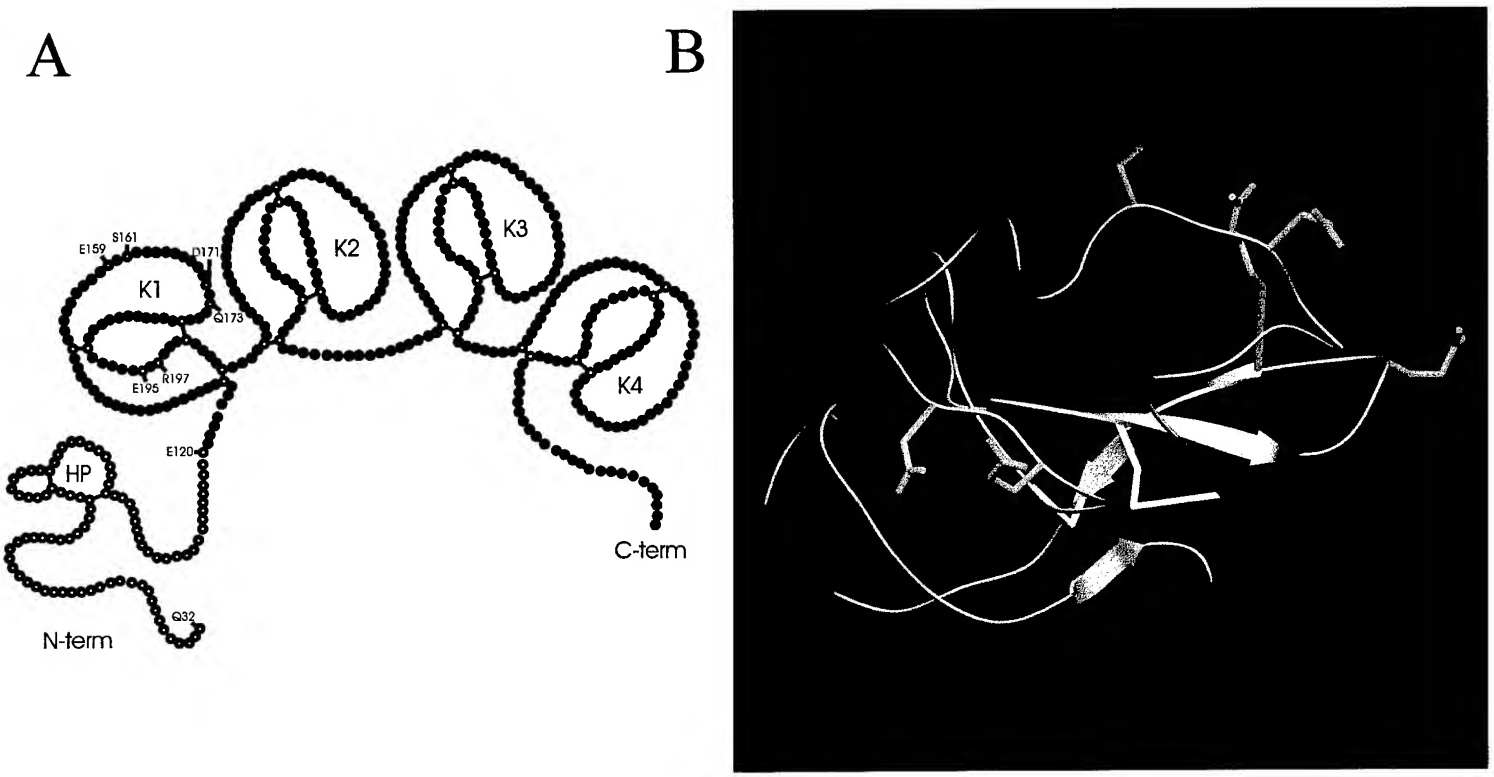
Activation of Rac1 stimulates HGF promoter activity in SP1 cells

Panel A) SP1 cells were co-transfected with 2.7 *HGF*-luc and SRC-RF (DN c-Src), or Rac1 L61 (activated Rac1), or both SRC-RF and Rac1 L61, as described in Fig. 12A. All groups were co-transfected with pSG5-βgal for normalization. After 24 h, cells were lysed; luciferase and β-galactosidase activities were determined, and mean relative luciferase activity was normalized to control (without Rac V14) cells. Bars indicate S.D. of triplicates.

Panel B) SP1 cells were transfected with 2.5 *HGF*-luc or Δ1 *HGF*-luc (lacking the c-Src responsive region) and pSG5-βgal, as in Figure 3. An activated Rac1 mutant (Rac1 V14) was co-transfected into cells from each group. After 24 h, cells were lysed; luciferase and β-galactosidase activities were determined, and mean relative luciferase activity was normalized to control cells. Bars indicate S.D. of triplicates.

The results show that Rac1-dependent induction of HGF promoter activity requires at least in part the c-Src/Stat3 responsive promoter sequence (see Fig. 14A).

Figure 17



Structure of HGF

Panel A: α-chain of HGF. The hairpin loop (HP), the four kringle domains (K1, K2, K3, K4) are indicated in the diagram. Two mutation-sensitive patches were identified in the K1 domain. The first patch consists of amino acids E159, S161, E195, R197, all of which are predicted to be close to each other in the tertiary structure of K1. The second patch, on the oposite side of K1, consists of D171 and Q173. A peptide in the N-terminal domain (Q32-E120) was used for immunization of rabbits.

Panel B: Modelled K1 domain of the alpha chain of HGF. The backbone is illustrated in ribbon representation and the side chains implicated in Met binding are shown. (Modelled by Dr. Z. Jia, Dept. of Biochem.)

PROTEIN EXPRESSION AND PURIFICATION 7, 329–333 (1996)
ARTICLE NO. 0047

Appendix I

Hepatocyte Growth Factor (HGF) Is a Copper-Binding Protein: A Facile Probe for Purification of HGF by Immobilized Cu(II)-Affinity Chromatography[1]

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Received August 21, 1995, and in revised form December 8, 1995

Hepatocyte growth factor (HGF) is a multifunctional protein expressed in a variety of cell types and tissues. Here we describe a novel one-step method to separate and identify HGF, based on a unique interaction between HGF and Cu(II). Conditioned medium (CM) from mouse 3T3-L1 adipocytes which contains HGF or purified human recombinant HGF was used for analysis. Mouse 3T3-L1 adipocyte CM was applied to a Cu(II)-affinity column and rinsed with equilibration buffer. HGF was then eluted with 10 mM imidazole. Fractions eluted from the column were analyzed by SDS–PAGE. Analysis by silver staining revealed an 85-kDa protein. Further analysis by Western blotting with polyclonal anti-HGF IgG demonstrated that this protein corresponded to HGF. Human recombinant HGF, when applied to a Cu(II)-affinity column, showed a stronger affinity to Cu(II) than did mouse HGF. Human recombinant HGF was not eluted from the Cu(II) column with either 10 or 20 mM imidazole; however, it was readily eluted with 40 mM imidazole. The percentages of recovery of both human and mouse HGF were greater than 90%. Both mouse HGF and human recombinant HGF eluted from the Cu(II)-affinity column retained their biological activity as measured by HGF-induced cell proliferation of Mv1Lu cells. Our findings provide the first evidence that HGF is a copper-binding protein and that a Cu(II)-affinity column can be used for efficient one-step purification of biologically active HGF. © 1996 Academic Press, Inc.

Hepatocyte growth factor (HGF) is a heterodimeric glycoprotein composed of two subunits, an α chain (69 kDa) and a β chain (34 kDa) (1), and exhibits pleiotropic functions. HGF stimulates DNA synthesis in various cell types including human and mouse mammary epithelial cells (2,3). HGF is also a morphogen that induces epithelial tubular formation and angiogenesis, a process that forms cords and tubes of endothelial cells *in vitro* (4). In addition to its mitogenic and morphogenic functions, HGF also induces epithelial cell motility (5,6). The receptor for HGF was identified as the product of the *met* proto-oncogene (7,8) and is involved in the transduction of cell regulatory signals in response to HGF (8,9).

Although a great deal of knowledge about *in vitro* functions of HGF has been demonstrated, at present the physiological role of HGF *in vivo* remains largely unknown. This is partly due to the lack of an efficient method for purification of preparative amounts of HGF from specific tissues and cell lines, and of sufficient quantities of HGF for functional studies *in vivo*. To date attempts to purify HGF have been costly, time consuming, and laborious: Platelets from more than 3000 rats were used to purify 60 μg of HGF by a three-step procedure involving cation-exchange chromatography, heparin–Sepharose affinity chromatography, and a C_4 column (10). More than 100 liters of rabbit serum or conditioned medium from MRC-5 fibroblasts was used to purify 50–100 μg of HGF; this purification involved heparin–Sepharose affinity chromatography, anion-exchange chromatography, and reversed-phase HPLC (11,2). Also, attempts to express HGF cDNA in the COS cell line resulted in relatively small yields (12,10).

In this study we demonstrate evidence that HGF is a copper-binding protein. HGF showed high-affinity

[1] This work was financially supported by the National Cancer Institute of Canada and the United States Army Medical Research and Materiel Command (USAMRMC) Grant DAMD 17-94-J-4407.

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binding to copper when an immobilized Cu(II)-affinity column was used. This unique interaction between HGF and Cu(II) provides a novel method for efficient one-step purification of HGF and HGF-related proteins.

MATERIALS AND METHODS

Materials. The following chemicals were obtained from Fisher Scientific Co. (Ottawa, Canada): imidazole, EDTA, Na_2HPO_4, and $CuSO_4$. Iminodiacetic acid coupled to epoxy-activated Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Rabbit anti-sheep IgG conjugated to horseradish peroxidase was from Bio/Can Scientific (Mississauga, Canada). ECL and Hyperfilm were from Amersham (Oakville, Canada). Silver staining reagents and nitrocellulose membrane were from Bio-Rad (Mississauga, Canada). Human recombinant HGF and affinity-purified sheep anti-human HGF IgG, which reacts with both human and mouse HGF, were kindly provided by Dr. R. Schwall (Genentech, Inc., San Francisco, CA). Conditioned medium (CM) from 3T3-L1 adipocytes was collected following incubation of cells for 24 h in serum-free DMEM as described previously (3). Mv1Lu cells are a mink epithelial cell line obtained from ATCC (Rockville, MA).

Cu(II)-immobilized affinity chromatography. Cu(II)-affinity chromatography was performed with iminodiacetic acid coupled to epoxy-activated Sepharose 6B (Pharmacia) (1 ml packed volume) according to the manufacturer's instructions. The column was loaded with 1 ml $CuSO_4$ (0.1 M) and was equilibrated with an equilibration buffer (10 ml) containing 0.02 M Na_2HPO_4, 1 mM imidazole, and 1 M NaCl (pH 7.2). 3T3-L1 adipocyte CM as a source of mouse HGF or purified recombinant human HGF was mixed with equilibration buffer and directly loaded onto the column; the column was then washed with the equilibration buffer (10 ml). Mouse HGF and recombinant human HGF were eluted at a flow rate of 1 ml/2 min with the equilibration buffer plus 10 and 40 mM imidazole, respectively. Fractions (10 ml) were collected and subsequently subjected to biochemical and functional analysis.

Western blotting. Proteins were separated by electrophoresis on 10% SDS–PAGE and transferred electrophoretically to a nitrocellulose membrane. The nitrocellulose membrane was incubated with sheep anti-HGF IgG (1:1000) and was visualized by successive incubations with rabbit anti-sheep IgG conjugated to horseradish peroxidase (1:5000). The ECL kit (Amersham) was used to detect the protein.

SDS–PAGE and silver staining. The protein samples were subjected to 10% SDS–PAGE. The proteins were visualized by a silver stain kit (Bio-Rad) which was used according to the manufacturer's instructions.

Cell proliferation assay. Mv1Lu cells were plated at 2×10^4 cells per well in 24-well plates under the various conditions indicated. DNA synthesis was measured at 2 days by adding 0.2 μCi [^{3}H]thymidine (Amersham) at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (Flow Laboratories), and [^{3}H]thymidine incorporation was measured in a scintillation counter (Beckman). Results are expressed as the mean cpm/well ± SD of triplicates.

RESULTS AND DISCUSSION

Immobilized metal-affinity chromatography, also known as metal chelate-affinity chromatography, has been used to identify interactions between certain proteins and divalent cations (13–15). Using immobilized Cu(II)-affinity chromatography, we demonstrated that HGF bound with high affinity to a Cu(II)-charged column. Our initial experiments demonstrated that retention of HGF on the column containing iminodiacetic acid coupled to epoxy-activated Sepharose 6B matrices required the presence of immobilized Cu(II): HGF was not eluted under typical elution conditions (0.02 M Na_2HPO_4 alone or plus 1 M NaCl and 1 mM imidazole). Instead, efficient elution of HGF required the presence of higher concentrations of imidazole, a specific competitor for bound copper, or EDTA, a chelator of divalent cations, in the elution buffer (data not shown). These results indicated that HGF bound to Cu(II) with high affinity and/or that multiple sites on the protein combined to form a highly stable interaction with immobilized Cu(II). We therefore decided to take advantage of this unique interaction between HGF and Cu(II) to purify HGF from physiological samples.

Previously we have demonstrated that mouse mammary 3T3-L1 adipocytes secrete biologically active HGF at high levels (3). In this study, 3T3-L1 adipocyte CM was used as a source of mouse HGF. 3T3-L1 adipocyte CM was concentrated and loaded directly onto a Cu(II)-affinity column. The column was rinsed with equilibration buffer (0.02 M Na_2HPO_4, 1 mM imidazole, and 1 M NaCl), and protein samples were eluted from the column with increasing concentrations of imidazole. A base amount of imidazole (1 mM) was added to the equilibration buffer to avoid nonspecific interactions of proteins present in the 3T3-L1 adipocyte CM. Aliquots of the eluates were analyzed by SDS–PAGE under nonreducing conditions followed by silver staining (Fig. 1A). Identical samples were also analyzed by Western blot analysis with anti-HGF IgG (Fig. 1B). The results revealed that HGF bound Cu(II) with high affinity and was not eluted from the column with the equilibration buffer (0.02 M Na_2HPO_4, 1 M NaCl, and 1 mM imidazole) or the equilibration buffer plus 5 mM

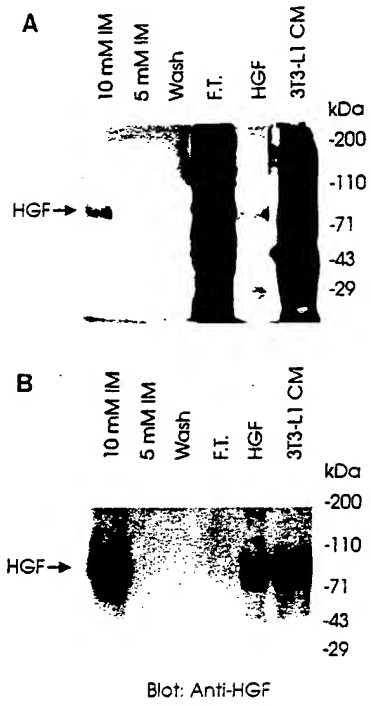

FIG. 1. Purification of mouse HGF from 3T3-L1 adipocyte CM. 3T3-L1 adipocyte CM (40 ml) was concentrated to 2 ml using Centricon 10 tubes and loaded onto a Cu(II)-affinity column. The column was rinsed with equilibration buffer (10 ml). Protein samples were sequentially eluted in 10-ml fractions with equilibration buffer plus 5 mM imidazole (5 mM IM) and 10 mM imidazole (10 mM IM). After concentration of eluates, aliquots corresponding to half of each fraction were separated by 10% SDS–PAGE (nonreducing conditions) and subjected to (A) silver staining and (B) Western blot analysis with anti-HGF IgG. Protein molecular weight standards are shown on the right. Tracks from right: 3T3-L1 CM (20-ml equivalent), HGF (0.05 μg), flowthrough (F.T.), wash, and 5 and 10 mM IM fractions.

imidazole. Efficient elution from the column of mouse HGF protein with an apparent molecular weight of 85 kDa was achieved with 10 mM imidazole (Figs. 1A and 1B). The flowthrough and fractions eluted with 5 mM imidazole did not contain any HGF as analyzed by Western blot analysis (Fig. 1B). However, non-copper-binding proteins readily came off the column in the flowthrough (Fig. 1A). Based on protein concentrations, the purification of HGF was at least 28-fold (Table 1). The same procedures have been successfully used to purify HGF from CM of a murine mammary carcinoma cell line, SP1 (data not shown).

We also evaluated the affinity of recombinant human HGF binding to the Cu(II)-affinity column. Interestingly, analysis of the eluates by silver staining revealed that recombinant human HGF bound Cu(II) with an even higher affinity than mouse HGF, and was not eluted from the column with the flowthrough, wash (equilibration buffer), or equilibration buffer containing either 10 (data not shown) or 20 mM imidazole

TABLE 1

Recovery of HGF Purified from 3T3-L1 Adipocyte Conditioned Medium

Fraction	Volume	Total protein (mg)[a]	Recovery[b]	Purity (fold)[a]
Original material	40 ml	24.6	100%	1
10 mM IM eluate	10 ml	0.8	95%	28

[a] 3T3-L1 adipocyte conditioned medium was separated on a Cu(II)-affinity column as described for Fig. 1A. The amount of protein in each fraction was determined using spectrophotometry at 280 nm and the fold purification of HGF protein eluted at 10 mM imidazole compared to total protein in the original sample was calculated.

[b] The percentage of recovery of HGF was determined by comparing the density of the protein bands corresponding to HGF in the sample eluted with 10 mM imidazole and in the original sample in Fig. 1B.

(Fig. 2). However, elution of recombinant human HGF from the column was effectively achieved with equilibration buffer containing 40 mM imidazole (Fig. 2) or 5 mM EDTA (data not shown). Subsequent elutions with equilibration buffer containing 80 mM imidazole or 5 mM EDTA yielded no additional HGF. The percentages of recovery of both mouse HGF and recombinant human HGF from the column were greater than 90% (Figs. 1 and 3 and Table 1). The difference observed in the affinity of human and mouse HGF for Cu(II) may be due to the minor differences in the composition of primary amino acid sequences between human and mouse HGF. The amino acid sequences of HGF appear to be highly conserved across species and the overall amino acid identity of mouse and human HGF is about 90% (16). The 10% differences in amino acid sequences

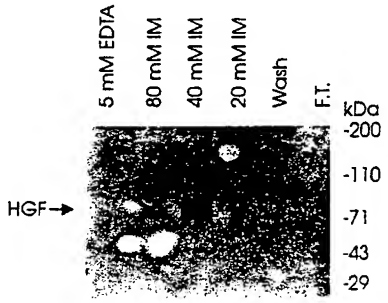

FIG. 2. Detection of human recombinant HGF eluted from a Cu(II)-affinity column. Purified human recombinant HGF (0.1 μg) was applied to a Cu(II)-affinity column. The flowthrough (F.T.) was collected and the column was rinsed with equilibration buffer (Wash). Protein samples were sequentially eluted with equilibration buffer containing 20 mM imidazole (20 mM IM), 40 mM imidazole (40 mM IM), 80 mM imidazole (80 mM IM), or 5 mM EDTA. The fractions were concentrated and aliquots corresponding to half of each fraction were subjected to silver staining as for Fig. 1. Protein molecular weight standards are shown on the right.

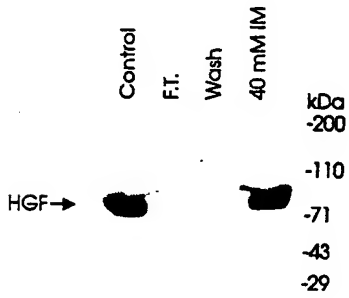

FIG. 3. Evaluation of efficiency of recovery of recombinant human HGF from a Cu(II)-affinity column. Purified human recombinant HGF (0.1 μg) was applied to a Cu(II)-affinity column. The flowthrough (F.T.) was collected and the column was rinsed with equilibration buffer (Wash). Protein samples were subsequently eluted with equilibration buffer containing 40 mM imidazole (40 mM IM). After concentration of eluates, half of each fraction or 0.05 μg of HGF (control) was loaded into each well and separated by 10% SDS–PAGE (nonreducing conditions) and subjected to silver staining. Protein molecular weight standards are shown on the right.

or posttranslational glycosylation of HGF may be reasons for such differences. Positively charged amino acids in particular histidine residues are most likely involved in mediating binding of HGF with copper. Identification of amino acids responsible for this interaction is currently being investigated. Overall, these results clearly indicate that both mouse and human HGF bind Cu(II) with high affinity, and/or that multiple sites on the protein combined to form a highly stable interaction with immobilized Cu(II).

Both mouse HGF and recombinant human HGF eluted from the column also showed biological activity in a cell proliferation assay (Figs. 4A and 4B). Aliquots of the eluates were assayed for growth factor activity by measurement of DNA synthesis in Mv1Lu cells. The mitogenic activity (100%) of 3T3-L1 adipocyte CM [previously shown to be HGF-induced (3)] and of recombinant human HGF was recovered in the fractions eluted with 10 and 40 mM imidazole, respectively, but not in the flowthrough (Figs. 4A and 4B). These results suggest that HGF was eluted specifically from the Cu(II)-affinity column with imidazole and is biologically active.

It has been demonstrated that exposure of some growth factors such as fibroblast growth factor (FGF) 1 and FGF2 to copper results in formation of intermolecular disulfide bonds by copper-induced oxidation of sulfhydryl residues of FGFs and subsequent loss of biological activity (17). Our results demonstrated that both mouse and human HGF eluted from a Cu(II)-affinity column retained biological activity and that no dimerization of HGF was detected in silver staining and Western blot analysis. Overall, these results indicate that interaction between HGF and copper is not the result of copper-induced formation of intermolecular disulfide bonds in the HGF molecule.

Our findings provide the first evidence that HGF is a copper-binding protein and that a Cu(II)-affinity column can be used for efficient purification of biologically

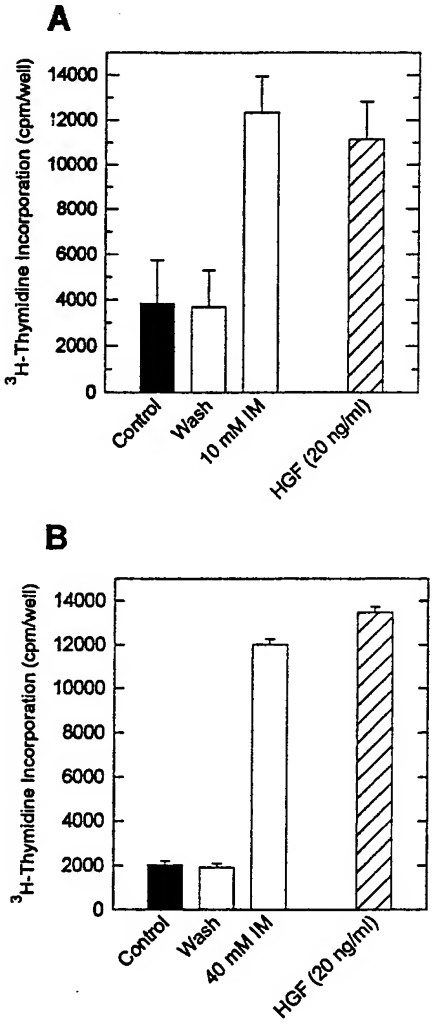

FIG. 4. Evaluation of biological activity of mouse and human recombinant HGF eluted from a Cu(II)-affinity column. Aliquots of the eluates of 3T3-L1 adipocyte CM and of human recombinant HGF from a Cu(II)-affinity column were used for a proliferation assay with Mv1Lu cells. (A) Biological activity of mouse HGF eluted from the column. Cells were plated (2×10^4/well) in serum-free DMEM in the absence of eluates (control) or in the presence of eluates (one-sixth volume per well) washed with equilibration buffer alone (Wash) or equilibration buffer plus 10 mM imidazole (10 mM IM) or in the presence of 20 ng recombinant human HGF. (B) Biological activity of recombinant human HGF eluted from the column. Cells were plated (2×10^4/well) in serum-free DMEM in the absence of eluates (control) or in the presence of eluates (one-sixth volume per well) washed with equilibration buffer alone (Wash) or equilibration buffer plus 40 mM imidazole (40 mM IM) or in the presence of 20 ng recombinant human HGF. DNA synthesis was measured as incorporation of [^{3}H]thymidine (0.2 μCi/well). The results are expressed as the mean cpm/well ± SD of triplicates.

active HGF. This method provides a simple, inexpensive, and novel method to purify HGF in large quantities. Our results also raise the possibility that copper-binding properties of HGF may play a role in HGF functions.

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Appendix II

IDENTIFICATION OF PARACRINE AND AUTOCRINE HEPATOCYTE GROWTH FACTOR LOOPS IN NON-SMALL CELL LUNG CARCINOMAS

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Running title: HGF paracrine and possible autocrine loop in lung carcinomas

Keywords: HGF, Met, protein secretion, cell proliferation, Metastasis, lung cancer

Manuscript in preparation

ABSTRACT

Hepatocyte growth factor (HGF) is secreted from mesenchymal cells and stimulates multiple functions including mitogenesis, motogenesis and morphogenesis of epithelial cells in many tissue types. HGF shows increased expression in human non-small cell lung carcinomas (NSCLC), but its role in tumor progression is not clearly known. We have examined expression of HGF and its receptor, Met, in NSCLC cell lines derived from primary tumor and pleural effusions of lung cancer patients. Using semi-quantitative RT-PCR and western blotting, we showed that 6/6 NSCLC cell lines expressed Met mRNA, but only two cell lines expressed detectable Met protein, implying incomplete translation or processing of Met in some carcinomas. Similarly, all NSCLC cell lines expressed significant or trace levels of HGF mRNA as determined using RT-PCR with direct radiolabelling of products, and 4/6 cell lines expressed detectable HGF protein. In contrast, 12 small cell lung carcinoma cell lines examined showed no detetable HGF or Met protein. Two HGF-expressing cell lines showed constitutive tyrosine-phosphorylation of Met, consistent with establishment of a functional autocrine HGF loop. Under serum-free detached conditions, these cells showed a high level of cell survival, compared to an HGF-negative, Met-positive cell line. These findings indicate that activation of an HGF autocrine loop occurs in some NSCLC cell lines and is associated with sustained survival of detached carcinoma cells; although additional paracrine stimulation by HGF is required for a mitogenic response. Thus autocrine HGF loops may provide a survival stimulus during early stages of tumor progression in NSCLC.

INTRODUCTION

Growth factors which act in a paracrine or autocrine manner are important regulators of stromal-tumor interactions in both normal and malignant cell development. Hepatocyte growth factor (HGF) is secreted primarily from mesenchymal/stromal cells of many different tissues (1-3); whereas HGF receptor (Met) expression is detected in a variety of epithelial (and some non-epithelial) cells in a broad range of tissues (4). HGF is secreted as an inactive single-chain pro-HGF protein of 105 kDa (5). Pro-HGF is cleaved by serine proteinases at Arg494-Val495, and is converted to a heterodimeric mature HGF molecule consisting of disulfide-linked α (65 kDa) and β (30 kDa) chains of 463 and 234 amino acid residues, respectively (6). Processing of HGF is required for its biological activities. Several known serine proteinases including urokinase (7) and tissue-type plasminogen activator (8), a protease homologous to blood coagulation factor XII (9), HGF converting enzyme (10), and other related HGF activating proteases (11), have been shown to activate HGF. Its activity can also be affected by association with extracellular matrix proteins such as proteoglycans, e. g. heparin (12). The *met* proto-oncogene product, a member of the tyrosine kinase receptor family, has been identified as the HGF receptor (13). Ligand binding induces kinase activation and tyrosine phosphorylation of Met (14). A two tyrosine motif in the COOH terminal tail of Met acts as a multifunctional docking site for SH2 domain-containing transducer proteins, resulting in stimulation of HGF-induced functions (15; 16). Activation of Met by HGF affects a variety of epithelial cell functions including cell proliferation (17; 18), survival (19), differentiation (20), cell motility (21), invasion (21), and angiogenesis (22).

Met is frequently over-expressed or amplified in many types of human cancers including non-small cell lung carcinomas (NSCLC) (23), breast (24), ovarian (25), and colorectal carcinomas

(26), melanomas (27), and osteosarcomas (28). We (24) and others (29) have previously shown strong expression of HGF mRNA in regions of invasive human breast cancer compared to more heterogeneous, weak, expression in ductal carcinoma *in situ* and nonmalignant epithelium. HGF is also over-expressed and often activated in NSCLC tissues (30; 31), and some small cell lung carcinoma (SCLC) cell lines (32; 33). This high level of HGF expression correlates with poor survival of cancer patients (29; 34). In contrast, HGF expression in corresponding normal lung epithelium is low (31; 35; 36), although expression of HGF by some bronchial epithelial and carcinoma cells *in vitro* has been shown (33; 37). Together, these findings suggest that establishment of an HGF autocrine loop may provide a selective advantage for autonomous growth and metastasis of carcinoma cells. This view was further promoted by the demonstration that expression of HGF (38) or a constitutively active mutant form of Met (Tpr-Met) (39) in transgenic mice, or in transformed cell lines (40; 41), promotes formation of tumors and metastasis. Altered expression of functionally-active HGF in epithelial cells could occur at several levels including transcription, and post-translational modification by enzymatic processing.

As a first step to examine the role of paracrine and autocrine HGF loops in NSCLC, we have used semi-quantitative RT-PCR and western blotting to examine the expression of HGF and Met mRNA and protein in established NSCLC cell lines. HGF protein was purified from conditioned medium, using copper(II) affinity chromatography, based on the ability of HGF to bind to Cu(II) (42). The activity of HGF protein secreted from different carcinoma cell lines was assessed by its ability to induce tyrosine- phosphorylation of Met in the lung carcinoma cell line, A549. The level of tyrosine-phosphorylation of Met, cell survival, and DNA synthesis was compared under

conditions of paracrine and autocrine stimulation by HGF. Our results suggest differential effects of paracrine and autocrine stimulation by HGF in NSCLC cells.

MATERIAL AND METHODS

Antibodies. Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from BD Transduction Laboratories (San Jose, CA). Mouse anti-human Met IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-human HGF IgG were received from Genentech Inc. (San Francisco, CA).

Human lung carcinoma cell lines. Human NSCLC cell lines SW-900 (43), WT-E (45), SK-Luci-6 (46), QU-DB (47), BH-E (45), LC-T (48) were established from primary tumor or pleural fluids from lung cancer patients. In some experiments a nonmalignant human bronchial epithelial cell line (HBE) was also used (49). Cell lines were routinely grown in RPMI 1640 medium, supplemented with 7% fetal bovine serum, and were confirmed to be mycoplasma negative as previously described (50).

PCR primers. The primers were designed according to the sequences of HGF and Met from Genbank using PC/GENE computer software. Primers were engineered to detect HGF and Met cDNA in both human and mouse, but not the homologous family members MSP (51) and Ron (52), respectively. The primers for HGF amplification were: sense primer 5' - TGT CGC CAT CCC CTA TGC AG - 3' located from position 69 to 88, and antisense primer 5' -TCA ACT TCT GAA CAC TGA GG - 3' located from position 610 to 629. The PCR product for HGF PCR amplification is 560

bp long (Figure1). The primers for Met amplification were: sense primer 5' - CCA CTA CAA CAT GAG CAG CC - 3' located from position 351 to 371, and antisense primer 5' - CTC CCT GCA GGT TTT GAT GC - 3' located from position 541 to 560. The PCR product for Met PCR amplification is 206 bp long (data not shown). In addition, primers for two house-keeping genes were used: glucuronidase B (GUSB) (53) and transferrin receptor (obtained from Dr. J. Gerlach).

RT-PCR analysis. Total RNA from various NSCLC tissues and cell lines was extracted using TriZol reagent (Canadian Life Technologies, Burlington, ON)), and used in a reverse transcriptase reaction to produce cDNA using a First-Strand Synthesis Kit (Amashan-Pharmacia Biotech, Baie d'Urfe, Que). PCR reactions were carried out without, or with, direct labelling of primers with [α-^{32}P]dATP (Mandel Scientific, Guelph, ON). cDNA (50 ng) was added to each 10 μl reaction, which also contained 20 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$. Oligonucleotide primers specific for HGF and GUS B (internal control), or Met and transferrin receptor (internal control) were added as indicated. GUS B and transferrin were chosen as controls since their mRNA levels were found empirically to correspond to those of HGF and Met, respectively. The reaction was initiated by the addition of 1 U of Taq polymerase (Canadian Life Technologies), and samples were then incubated at 95°C for 1 minute (denaturing), 55°C for 1 minute (annealing), 72°C for 1 minute (elongation). Unlabelled and labelled PCR was carried out with 25 cycles. The reaction products were resolved on a 1.5% agarose gel. Unlabelled PCR products were stained with ethidium bromide and visualized under UV light illumination. Labelled PCR products were measured in gel slices using a liquid scintillation counter.

Copper (II) affinity chromatography. Purification of HGF was carried out using Cu (II) affinity chromatography based on the Cu(II) binding ability of HGF (42). The principal of separation of HGF from biological samples by Cu(II) affinity chromatography is based on the fact that HGF has several cationic sequences (His-X-His) in the kringle domains of the HGF molecule. Five ml of each conditioned medium were loaded onto a 1 ml Cu(II) chelating column, which had been equilibrated with equilibration buffer (20 mM sodium phosphate, pH 7.2, 1 M NaCl, 1 mM imidazole). HGF protein bound specifically to the copper (II) affinity column, and unbound proteins were washed away with 15 column volumes of the same buffer. HGF protein was eluted using equilibration buffer containing 80 mM imidazole, and the eluant was concentrated using an Amicon Microcon-10 concentrator before being analyzed on 8% SDS PAGE under reducing conditions. Proteins were then transferred onto nitrocellulose membrane and probed with a sheep anti-human HGF antibody (Genentech Inc.). The bands were revealed using an ECL kit (Amersham Pharmacia Biotech).

Immunoprecipitation and western blot analysis. NSCLC cell lines were grown to 80% confluence and serum-starved for 24 hour. Cell lines were then rinsed with phosphate-buffered saline and lysed in a lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 minutes at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein from cell lysates were immunoprecipitated with the indicated antibodies at 4°C for 2 h. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, and subjected to 8% SDS-

PAGE under reducing conditions, followed by western blotting with the indicated antibodies. Immunoreactive bands were revealed using an ECL kit.

Met kinase assay. Cells were cultured to 80% confluence and serum starverd overnight. The cells were then treated without or with HGF (20ng/ml) for 20 min at 37 °C. The cell were lysed and extracts were normalized for protein concentration and precipitated with anti-Met IgG. The immunoprecipitates were washed twice with lysis buffer and once with kinase buffer containing 20 mM Pipes (pH 7.0), 10 mM $MnCl_2$ and 10 μM Na_3VO_4 and resuspended in cold kinase buffer. The reaction was initiated by addition of 10μCi [γ-^{32}P]ATP. The reaction was carried out at 30 °C for 10 min and stopped by adding sample buffer and boiling for 3 min. The samples were subjected to 8 % SDS-PAGE and the gel was treated with 1 M KOH for 30 min at 45 °C to hydrolyse Ser/Thr phosphorylation sites. The gel was then fixed in 45% MeOH and 10% acetic acid for 30 min at room temperature and dried. The autophosphorylation of Met was analyzed by a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Met activation Assay for HGF activity. The human lung carcinoma cell line, A549, which expresses Met, does not produce HGF protein in our system, was used as a read-out for Met activation. A549 cells were exposed for 30 min to conditioned media from various NSCLC cell lines, HGF (20 ng/ml), or medium alone. The cells were lysed and subjected to immunoprecipitation with anti-Met antibody. Immunoprecipitates were subjected to 8% SDS-PAGE under reducing conditions, and immunoblotting was carried out with anti-phosphotyrosine antibody or anti-Met antibody (loading control). Immunoreactive bands were revealed using an ECL kit.

Cell survival. Carcinoma cells were cultured in tissue culture plates and pre-starved overnight, harvested and seeded at a density of $2x10^4$ cells in 1.5 ml of RPMI 1640 medium containing 0.5 mg/ml BSA in 0.6% agar-coated 35 mm Corning non-tissue culture plates. After 24 h incubation at 37°C with indicated treatment, cells were collected and centrifuged in Eppendorf tubes (1000 rpm for 5 min), replated in a 96-well plate with 7% FBS/RPMI medium, and incubated at 37°C for 4 h. Cell survival was then measured using a colorimetric method based on the conversion of MTS tetrazolium to formazan (CellTiter aqueous Kit, Promega, Madison, WI).

DNA synthesis. Carcinoma cells ($1x10^4$) were incubated in triplicate in a 24 well plate for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 hours, 0.2 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) was added, and cells were incubated for an additional 24 hours period. Cells were harvested and aliquots of cells (1000 per well) were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (ICN, Costa Mesa, CA), and DNA synthesis was measured as incorporation of [^{3}H]thymidine in a scintillation counter (Beckman, Mississauga, ON, Canada).

RESULTS

Expression of HGF and Met mRNA in non-small cell lung carcinoma tissues and cell lines: The level of HGF and Met mRNA was first examined using RT-PCR analysis. The linearity of the PCR reaction was assessed to establish the optimal reaction conditions, and the reaction was performed

with internal controls using β glucuronidase (GUS B) for HGF, and transferrin receptor for Met. The PCR reaction was carried out with 25 cycles which was found to be linear. Human NSCLC cell lines SK-Luci-6, SW-900 and BH-E expressed significant levels of HGF mRNA detected using both unlabelled and labelled PCR; whereas WT-E, LC-T and QU-DB cell lines showed trace levels of HGF mRNA detected only using radiolabelled PCR (Figure 1 and Table I). A nonmalignant human bronchial epithelial cell line, HBE, also showed trace amounts of HGF mRNA expression. All cell lines tested showed detectable levels of Met mRNA expression (Table I).

Expression of HGF and Met protein in non-small cell lung carcinoma cell lines: To detect HGF protein secreted by carcinoma cell lines, we employed Cu (II) affinity chromatography (42) to isolate HGF from conditioned media. Western blotting of purified protein was carried out and putative HGF protein was detected with sheep anti-human HGF antisera which recognizes both α and β chains of the HGF molecule. Conditioned media from NSCLC cell lines SW-900, WT-E, SK-Luci-6 and BH-E all showed detectable expression of HGF protein. However, conditioned media from NSCLC cell lines QU-DB, and LC-T showed no detectable HGF protein (Figure 2). Met protein was detected in cell lysates of two HGF-secreting NSCLC cell lines (WT-E and SW-900); but not in the remaining cell lines (Table I). In contrast, HGF and Met protein were not expressed in a series of 12 small cell lung carcinomas tested (Table II).

HGF activity from conditioned media (CM) of non-small cell lung carcinoma cells: To assess the activity of HGF in CM from carcinoma cell lines, we tested the ability of CMs to activate Met in A549 carcinoma cells (which are Met positive, HGF negative). A control showed strong tyrosine-

phosphorylation of Met in A549 cells incubated with exogenous HGF (40 μg/ml), compared to cells incubated in media alone. Three out of four HGF-containing conditioned media from human NSCLC cell lines tested stimulated tyrosine phosphorylation of Met in A549 cells (Figure 3). These results indicate that the majority of HGF-producing NSCLC cell lines tested secrete active HGF protein.

Status of tyrosine-phosphorylation of Met in non-small cell lung carcinoma cell lines: A prediction from these studies is that carcinoma cell lines that express both active HGF and Met would show constitutive activation of Met, consistent with the establishment of an HGF autocrine loop in these cells. To test this possibility, we examined the tyrosine-phosphorylation level of Met in two NSCLC cell lines (WT-E and SW-900) without, or with, incubation with HGF (20 ng/ml), using western blot analysis. Both cell lines which co-express HGF and Met, showed significant tyrosine-phosphorylation of Met even without treatment with exogenous HGF. These two cell lines were also assayed for Met kinase activity *in vitro* and they showed consititutively active Met without HGF stimulation (Figure 4).

Cell survival under anchorage-independent conditions: To test the biological function of putative paracrine versus autocrine activation of Met in NSCLC cell lines, we examined cell survival in A549, SW-900 and WT-E cell lines. SW-900 and WT-E cells, with autocrine expression of HGF and sustained tyrosine-phosphorylation of Met, consistently showed high level of cell survival under nonadherent serum-starved conditions; whereas A549 cells which express Met but not HGF showed a reduced survival response (Figure 5A). In addition, SW-900 and WT-E cells, not A549 cells,

showed a sustained high level of Met phosphorylation when cells were kept in suspension, which correlated with the cell survival under anchorage-independent conditions (Figure 5B).

DNA synthesis and phosphorylation state of ERK in non-small cell lung carcinoma cell lines: We also examined DNA synthesis in A549, SW-900 and WT-E cell lines. Interestingly, the base level of DNA synthesis in the absence of exogenous HGF was low in all cell lines, regardless of the level or activity of secreted HGF or Met kinase activity, and they showed strong paracrine stimulation by HGF of DNA synthesis. As a control, LC-T (HGF and Met negative) showed no stimulation. (Figure 6 and Table I). The phosphorylation of ERK1/2 was increased by addition of exogenous HGF to the cells (Figure 7). Altogether, these results suggest that increased cell survival, but not DNA synthesis, correlated with the expression of HGF and autophosphorylation of Met at tyrosine residues in NSCLC cell lines.

DISCUSSION

A broad range of genetic markers have ben associated with invasive lung cancer including amplification of EGF receptor (55), K-ras point mutations (56), and dominant negative mutations of p53 (57). However, to date there are no reliable genetic markers for premalignant changes in lung epithelia. Previous studies have shown that HGF is over-expressed and often activated in the majority of NSCLC tissues compared to adjacent normal lung tissue (30,31), and this high level of HGF expression has been identified as a possible independent predictor of poor survival of lung cancer patients (30). Met is also frequently over-expressed in NSCLC, particularly in regions of

invasive adenocarcinomas and large cell undifferentiated carcinomas (23). Earlier studies with NSCLC cell lines have demonstrated autocrine HGF expression and function in a lung bronchial epithelial and carcinoma cell line (33), and autocrine stimulation of motility by a two-kringle variant of HGF in a small cell lung carcinoma cell line (37). More recent results from Yi *et al.* (32) have shown expression of HGF mRNA in a broad range of NSCLC cell lines, however the expression of active HGF protein and existence of possible paracrine and autocrine HGF loops was not fully investigated in the study.

To assess the presence of possible paracrine and autocrine HGF loops, we first examined HGF and Met expression and function in six NSCLC cell lines established from primary tumors and pleural effusions (See Table I). Our study involved a combined analysis of HGF and Met mRNA and protein expression, and functional roles of paracrine and autocrine activation of Met. The results showed that all of the NSCLC cell lines tested expressed detectable HGF mRNA, of which four cell lines secreted HGF protein. Western blot analysis of proteins resolved by SDS-PAGE under reducing conditions showed bands corresponding to both pro-HGF (100 kDa) and mature alpha chain (65 kDa). Similarly, Met mRNA was detected in all NSCLCs, however only two cell lines (SW-900 and WT-E) expressed significant levels of Met protein. The differences in Met and HGF mRNA versus protein detected could represent different sensitivities of the assays, or differences in post-transcriptional or postranslational steps in the expression of HGF and Met in NSCLC cells. Conditioned media from only three of the HGF-producing cell lines (SW-900, SK-Luci-6, and BH-E) showed biologically active as determined by the ability to stimulate tyrosine-phosphorylation of Met in A549 cells (Met postive, HGF negative). The lack of detectable HGF activity in HGF-containing CM from WT-E cells could reflect sensitivity of the assay, or inactivation of HGF due to association with extra cellular matrix proteins (12) or degradation (58).

Although expression of secreted HGF and Met was detected in NSCLC cell lines derived from adenocarcinomas, squamous cell carcinomas, and large cell anaplastic carcinomas, the number of cell lines examined was insufficient for statistical correlation to specific NSCLC histotypes. In addition, some NSCLC cell lines expressed HGF, but not Met, and some expressed neither HGF nor Met. In contrast, no expression of HGF or Met was detected in 12 small cell lung carcinoma cell lines examined (data not shown). Together these findings imply that both paracrine and autocrine HGF loops may be important in development of NSCLC.

Two NSCLC cell lines (SW-900 and WT-E) which express high levels of Met, also secreted HGF protein, demonstrating the existence of a putative autocrine HGF loop in a significant proportion of NSCLC cells. Our findings further showed tyrosine-phosphorylation of Met and increased Met kinase activity in both cell lines, suggesting the presence of an autocrine HGF loop in these cells. Interestingly, conditioned media from WT-E cells showed no detectable HGF activity, although HGF protein was evident (Figure 2). Thus, WT-E cells , which showed a high level of activated Met, may exhibit intracrine activation of Met via association with the cytoplasmic pool of HGF inside the cell, as has been shown for fibroblast growth factor receptor (60) and epidermal growth factor receptor (61). Alternatively, activity of soluble HGF may be blocked by association with proteoglycans such as heparin sulphate (18).

To further assess the functional role of paracrine and autocrine activation of Met in NSCLC cells, we determined the cell survival and DNA synthesis responses without, or with, addition of exogenous HGF. Interestingly, all Met-expressing NSCLC cell lines required paracrine stimulation with HGF for an optimal proliferation response, regardless of the presence of an autocrine HGF loop. Two NSCLC cell lines (SW-900 and WT-E) which express an autocrine HGF loop showed a sustained high level of Met tyrosine-phosphorylation and cell survival under nonadherent conditions.

In an independent study, Yi *et al*, (32) showed that NSCLC cell lines expressing HGF mRNA exhibited paracrine, but not autocrine, stimulation by HGF of DNA synthesis; however the level of constitutive activation of Met was not assessed in their study. Our results indicate that autocrine activation of Met is sufficient to sustain survival of NSCLC cells, whereas additional paracrine stimulation with HGF is required to stimulate DNA synthesis.

In summary, our panel of NSCLC cell lines represent different stages of tumor progression including acquisition of an autocrine HGF loop, and concomitant loss of anchorage requirement for cell survival and growth. Our findings further raise the possibility that paracrine and autocrine stimulation of Met triggers different functional responses in NSCLC cells, possibly due to quantitatively or qualitatively different signalling patterns. Experiments are in progress to further assess the pattern of HGF-induced signalling and cell functions associated with paracrine versus autocrine HGF stimulation of NSCLC cells.

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TABLE I

Expression of HGF and Met in human Non-small cell lung carcinoma cell lines

Carcinoma Cell Lines	Histology	Origin	HGF			Met		HGF-induced DNA synthesis[c]
			mRNA	Protein	Activity	mRNA	Protein	
SW-900	squamous cell carcinoma	primary tumor	+[b]	+	+	+	+	+
WT-E	squamous cell carcinoma	pleural effusion	+/-	+	-	+	+	+
SK-Luci-6	large cell anaplastic	primary tumor	+	+	+	+	-	-
QU-DB	large cell anaplastic	primary tumor	+/-	-	-	+	-	-
BH-E	adenocarcinoma	pleural effusion	+	+	+	+	-	-
LC-T	adenocarcinoma	primary tumor	-	-	-	+	-	-

Legend:

a) See Materials and Methods for designation of Non-small cell lung carcinoma cell lines.

b) N/D, not determined; +, positive; +/-, trace amount; -, negative.

c) Cells ($1x10^4$) in triplicate were incubated in a 24 well plate for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 h, ^{3}H-Thymidine was added, and cells were incubated for a second 24 h period. DNA synthesis was measured as incorporation of ^{3}H-Thymidine.

TABLE 2

Expression of HGF and Met in human small cell lung carcinoma cell lines

Carcinoma Cell Lines[a]	Origin	HGF			Met	
		mRNA	Protein	Activity	mRNA	Protein
BK-T	primary tumor	-[b]	-	-	+	-
CK-A	needle aspiration	-	-	-	+	-
H69	pleural effusion	-	-	-	+	-
H128	pleural effusion	-	-	-	+	-
HA-E	pleural effusion	-	-	-	+	-
HG-E	pleural effusion	-	-	-	+	-
LG-T	lymph node biopsy	-	-	-	+	-
MM-1	pleural effusion	-	-	-	+	-
SHP-77	pleural effusion	-	-	-	+	-
SM-E	pleural effusion	-	-	-	+	-
SV-E	pleural effusion	-	-	-	+	-
YR-A	needle aspiration	-	-	-	+	-

Legend:

a) See Materials and Methods for designation of non-small cell lung carcinoma cell lines, and details of procedures for mRNA and protein analysis of HGF and Met.

b) N/D, not determined; +, positive; +/-, trace amount; -, negative.

FIGURE LEGENDS

Figure 1. Analysis of NSCLC cell lines: Total RNA from various NSCLC cell lines was extracted and used in a reverse transcription reaction to produce cDNA. A nonmalignant lung bronchial cell line, HBE (59), was used as a control. Primers specific for HGF and GUS B (as internal control) were added to the cDNA. Unlabelled (Panel A) and labelled (Panel B) PCR was carried out with 25 cycles, and the reaction products were analysed as described in Materials and Methods.

Figure 2. Detection of HGF protein in conditioned media of NSCLC cell lines: The presence of HGF protein in the conditioned media collected from different lung carcinoma cell lines was determined using copper (II) affinity chromatography to purify putative HGF from conditioned media. Fractions containing putative HGF were concentrated by Microcon concentrators, and were analysed by reducing SDS-PAGE, followed by western blotting with sheep anti-HGF antibody (Genentech). Immunoreactive bands were revealed by ECL. Arrows corresponding to pro-HGF and mature HGF are shown.

Figure 3. HGF activity in conditioned media from NSCLC cell lines: A549 cells which express Met but not HGF were prestarved before incubation with conditioned media from various cell lines. Controls consisted of cells incubated without, or with, HGF (40 ng/ml). After 30 min of incubation at 37°C, cells were washed with ice-cold PBS, lysed in lysis buffer, immunoprecipitated with rabbit anti-Met antibody. Immunoprecipitates were washed several times with lysis buffer before analysed by reducing SDS-PAGE. Proteins were analyzed using western blotting with anti-phosphotyrosine antibody (anti-PY) (upper) or with anti-Met antibody (lower).

Figure 4: Met is constitutively active in SW-900 and WT-E cells. A549, SW-900 and WT-E NSCLS cell lines were cultured to 80% confluence and serum-starved overnight. The cells were then treated

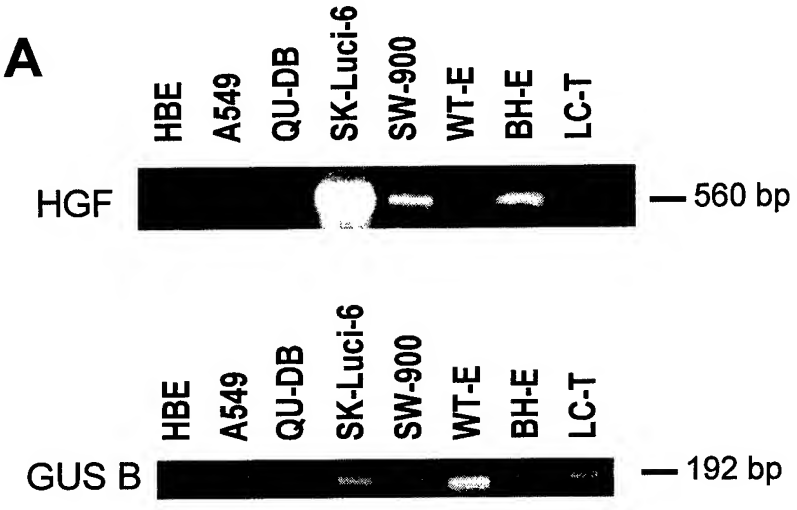
with HGF (20 ng/ml) for 20 min at 37°C and lysed. Clarified cell extracts were normalized for protein concentration and precipitated with anti-Met IgG. Panel A: Half of the immunoprecipitates were analysed by western blotting. The blot was probed with anti-PY antibody, and the bands were visualized with ECL reagents. The same blot was stripped and re-probed with anti-Met IgG as a loading control. Panel B: Half of the immunoprecipates were assayed for Met kinase activity *in vitro* as described in Materials and Methods. The signal densities were measured with a PhosphoImager and plotted relative to the control A549 cells without HGF (lower panel).

Figure 5: SW-900 and WT-E cells show a high level of survival and constitutive Met phosphorylation under anchorage-independent conditions. Panel A: A549, SW-900 and WT-E cells were serum-starved overnight, and seeded in suspension cultures with the treatments as indicated. After 24 h incubation at 37°C, cells were transferred to 96-well plates and surviving cells were measured with the MTS colorimetric assay as described in Materials and Methods. The results are expressed as mean ± range of duplicates, and are representative of two experiments. Panel B: The cells were serum-starved and put in suspension (S) or left on plates (A) for 4 h in 37°C. The cells were then lysed and cell extracts were immunoprecipitated with anti-Met IgG and analyzed by western blotting with anti-PY or anti-Met antibodies.

Figure 6. Exogenous HGF induces DNA synthesis in A549, SW-900 and WT-E cell lines: Cells were serum-starved overnight and subcultured in 96-well tissue culture plates (1000 cells per well) without, or with, HGF at the concentrations indicated, as described in the Materials and Methods. A control consisted of the LC-T cell line (HGF and Met negative). After 24 h, 0.2 μCi of [^{3}H]thymidine was added, and cells were incubated for an additional 24 h cells. Cells were then harvested, transferred to filters, and the incorporation of [^{3}H]thymidine was measured using a scintillation counter. Results are expressed as the mean cpm of quadruplicate wells ± S.D.

Figure 7: Exogenous HGF stimulates phosphorylation of ERK in A549, SW-900 and WT-E cells. The cells were cultured to 80% confluency and serum starved overnight. Cells were treated without, or with, HGF (20 ng/ml) for 20 min and then lysed. The cell extracts were analyzed by western blotting with anti-phosphor-ERK1/2 antibody. The same blot was stripped and re-probed with anti-ERK2 antibody to confirm equal protein loading between groups.

Figure 1


A
HBE
A549
QU-DB
SK-Luci-6
SW-900
WT-E
BH-E
LC-T
HGF
560 bp
HBE
A549
QU-DB
SK-Luci-6
SW-900
WT-E
BH-E
LC-T
GUS B
192 bp

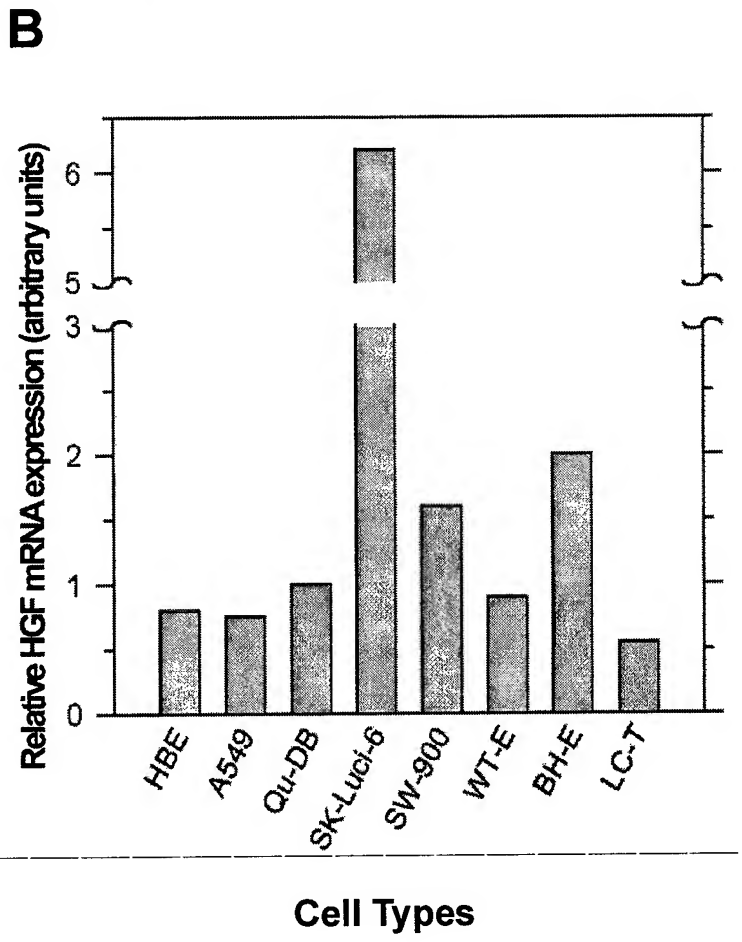

B
Relative HGF mRNA expression (arbitrary units)
0
1
2
3
5
6
HBE
A549
Qu-DB
SK-Luci-6
SW-900
WT-E
BH-E
LC-T
Cell Types

Figure 2

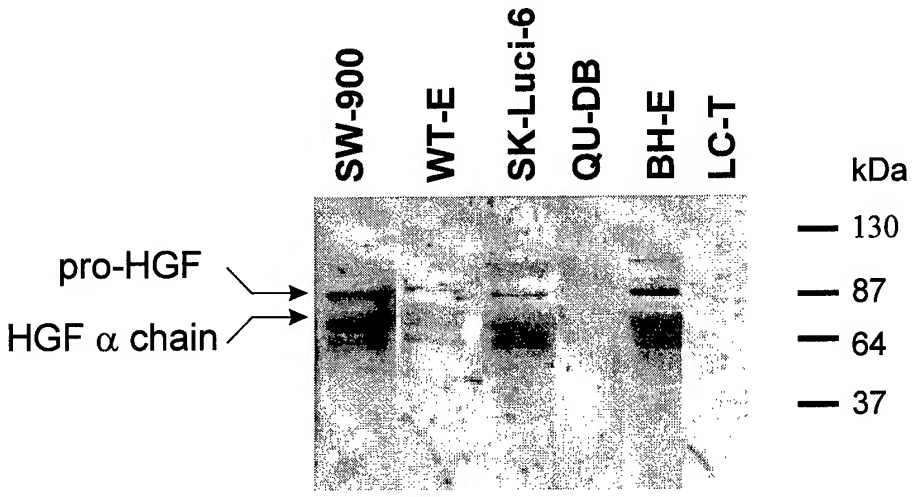
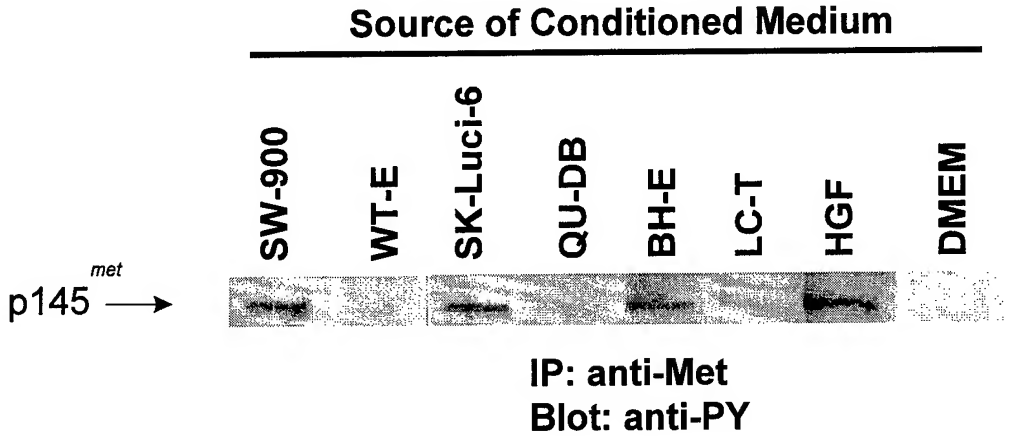

SW-900
WT-E
SK-Luci-6
QU-DB
BH-E
LC-T
kDa
130
87
64
37
pro-HGF
HGF α chain

Figure 3


Source of Conditioned Medium
SW-900
WT-E
SK-Luci-6
QU-DB
BH-E
LC-T
HGF
DMEM
met
p145
IP: anti-Met
Blot: anti-PY

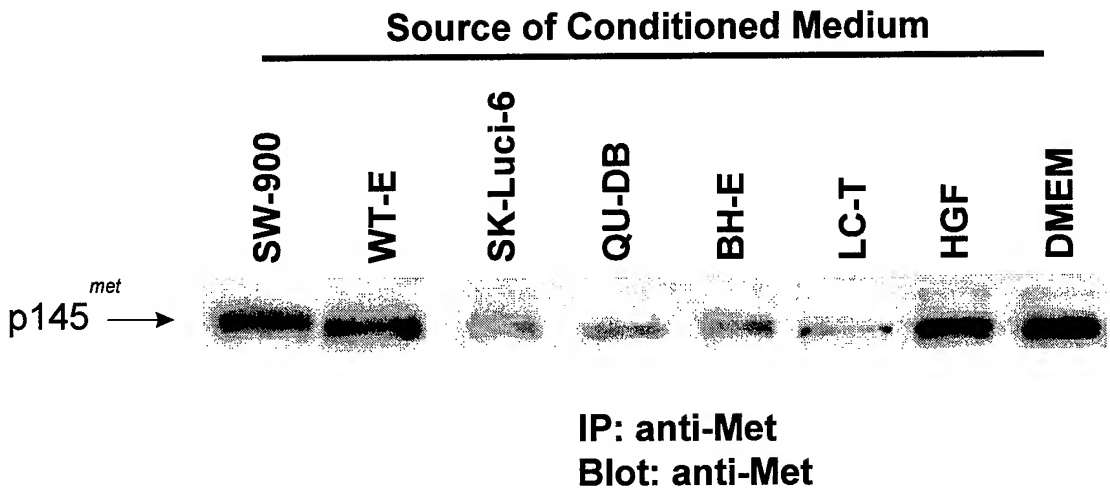

Source of Conditioned Medium
SW-900
WT-E
SK-Luci-6
QU-DB
BH-E
LC-T
HGF
DMEM
met
p145
IP: anti-Met
Blot: anti-Met

Figure 4

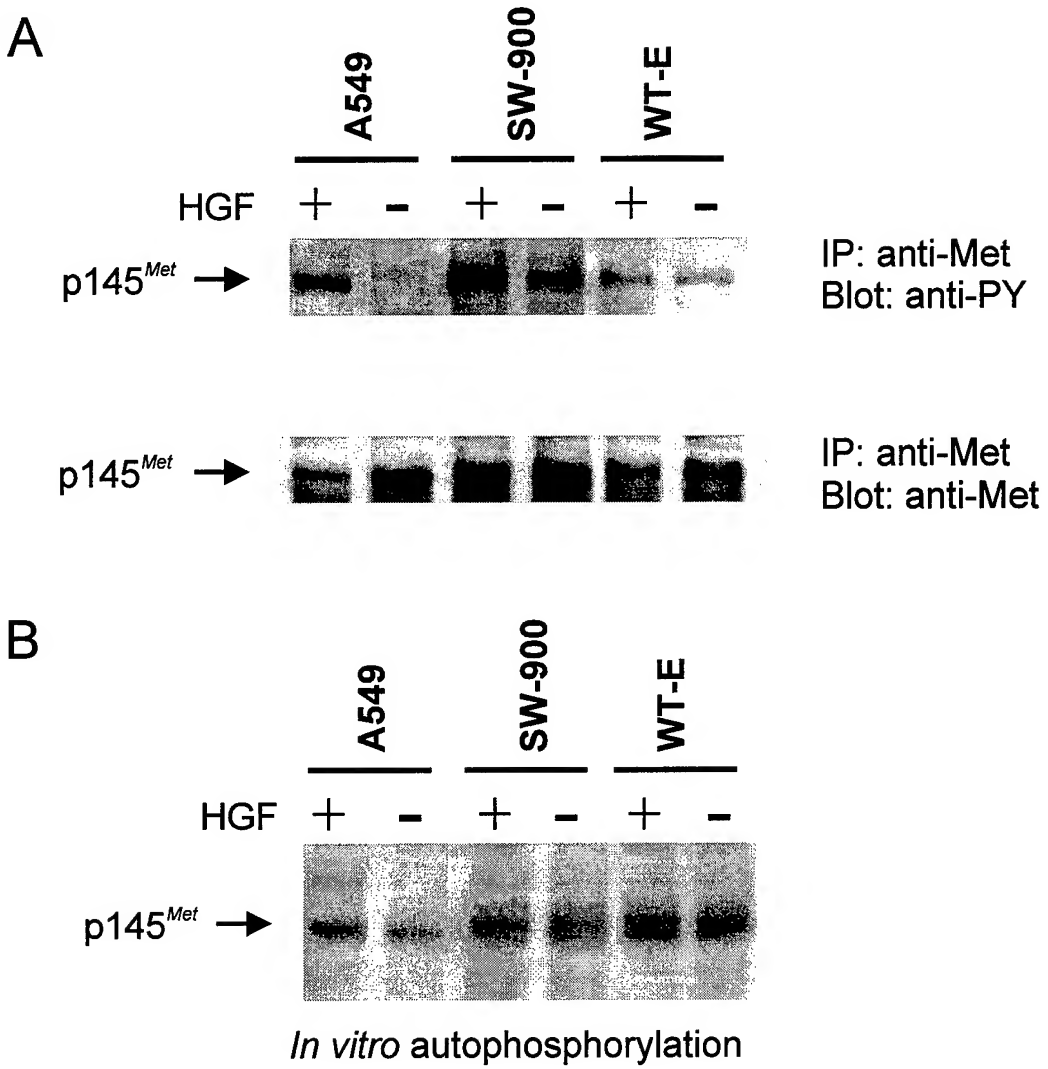

A
A549
SW-900
WT-E
HGF + - + - + -
p145Met
IP: anti-Met
Blot: anti-PY
p145Met
IP: anti-Met
Blot: anti-Met
B
A549
SW-900
WT-E
HGF + - + - + -
p145Met
In vitro autophosphorylation
Specific Met Kinase Activity (fold of control)
+ HGF
- HGF
0
1
2
3
4
A549
SW-900
WT-E
Cell Line

Figure 5

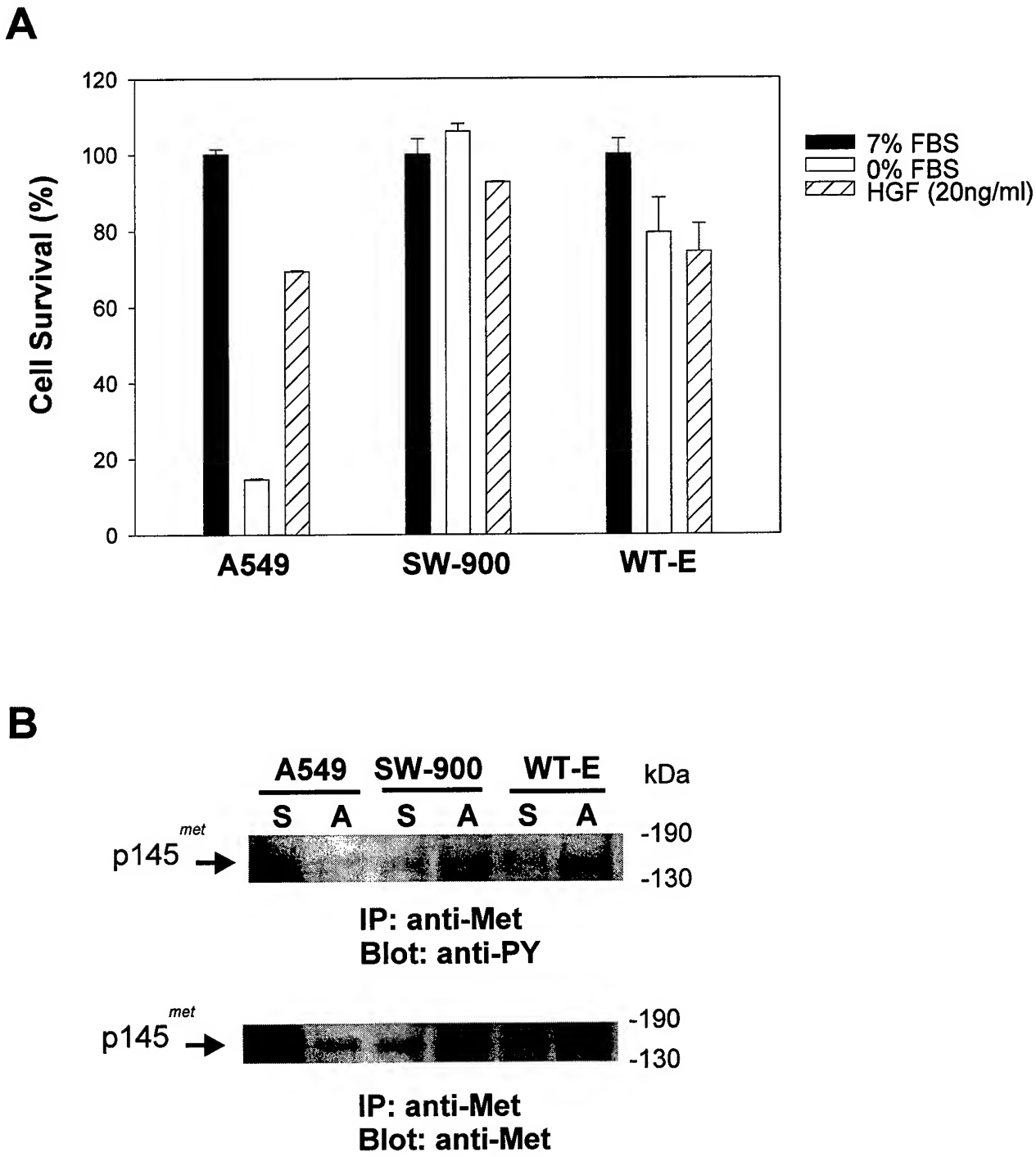

A
120
100
80
60
40
20
0
Cell Survival (%)
A549
SW-900
WT-E
7% FBS
0% FBS
HGF (20ng/ml)
B
A549
SW-900
WT-E
kDa
S A S A S A
p145 met
-190
-130
IP: anti-Met
Blot: anti-PY
p145 met
-190
-130
IP: anti-Met
Blot: anti-Met

Figure 6

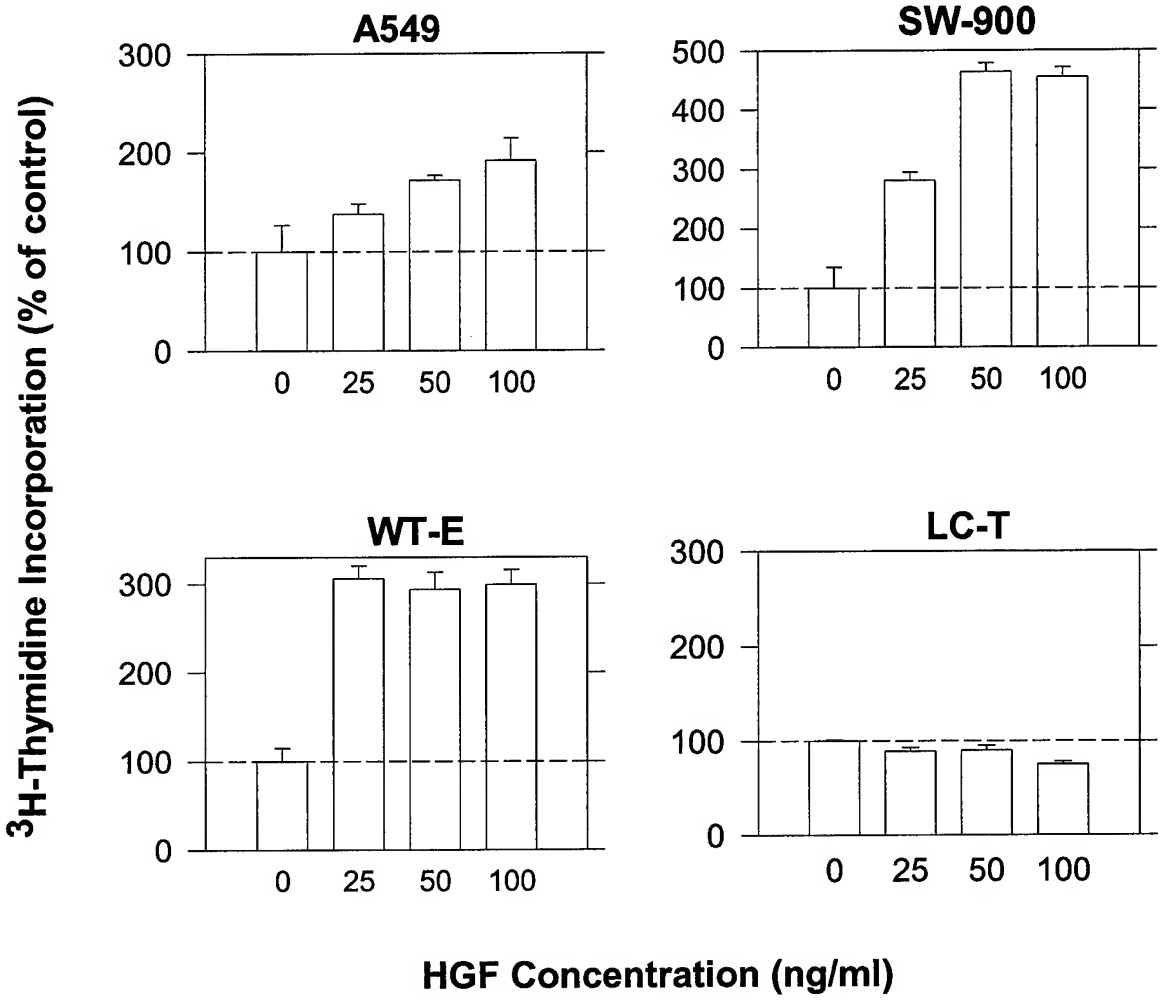
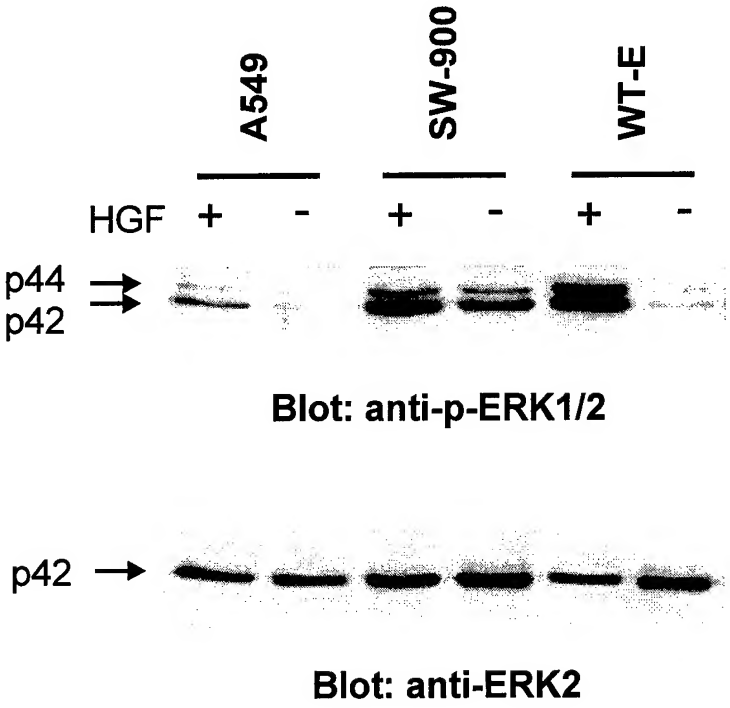

A549
SW-900
WT-E
LC-T
300
200
100
0
500
400
300
200
100
0
0 25 50 100
^{3}H-Thymidine Incorporation (% of control)
HGF Concentration (ng/ml)

Figure 7


A549
SW-900
WT-E
HGF + - + - + -
p44
p42
Blot: anti-p-ERK1/2
p42
Blot: anti-ERK2

Appendix III

Journal of Cellular Biochemistry 78:465–475 (2000)

Osteopontin-Induced, Integrin-Dependent Migration of Human Mammary Epithelial Cells Involves Activation of the Hepatocyte Growth Factor Receptor (Met)

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Abstract Osteopontin (OPN) is a secreted glycophosphoprotein which induces migration of mammary carcinoma cells, and has been implicated in the malignancy of breast carcinoma. Hepatocyte growth factor (HGF) induces cell migration of several mammary epithelial cell (MEC) lines, via activation of its cognate receptor (Met). This study examines the mechanism of OPN-induced MEC migration, in terms of the cell surface integrins involved and induction of the HGF/Met pathway. Three different MEC cell lines were used, representing different stages of tumor progression: 21PT, non-tumorigenic; 21NT, tumorigenic; non-metastatic; and MDA-MB-435, tumorigenic, highly metastatic. Human recombinant OPN was found to induce the migration of all three lines. OPN-induced migration of 21PT and 21NT cells was αvβ5 and β1-integrin dependent, and αvβ3-independent, while that of MDA-MB-435 cells was αvβ3-dependent. HGF also induced migration of all three cell lines, and a synergistic response was seen to HGF and OPN together. The increased migration response to OPN was found to be associated with an initial increase in Met kinase activity (within 30 min), followed by an increase in Met mRNA and protein expression. OPN-induced cell migration is thus mediated by different cell surface integrins in MEC lines representing different stages of progression, and involves activation of the HGF receptor, Met. J. Cell. Biochem. 78:465–475, 2000.

Key words: osteopontin (OPN); cell migration; integrin; hepatocyte growth factor (HGF); Met; mammary epithelial cells; breast cancer

Growth, migration, and differentiation of epithelial cells are known to be dependent upon integrin-mediated adhesion to extracellular matrix components [reviews in Assoain, 1997; Bissell, 1999; Gumbiner, 1996]. Similarly, these same cellular processes are also known to be influenced by a number of different growth factor pathways [reviews in Seedorf, 1995; Vande Woude et al., 1997; Heldin, 1998; Birchmeier, 1998]. Several recent studies have addressed the possibility of interactions between integrin and growth factor mediated pathways, with evidence emerging for both growth factor control of cell adhesion events [van der Voort et al., 1997; Weimar et al., 1997; Trusolino et al., 1998; Weimar et al., 1998], and conversely, for integrin-mediated cell adhesion phenomena influencing sensitivity to certain growth factors [Miyamoto et al., 1996; Brooks et al., 1997]. However, the mechanism and biological relevance of these growth factor-integrin interactions are not yet clear.

Our group has particular interest in the role of the secreted glycophosphoprotein OPN in the malignancy of breast cancer. We have found that OPN can induce cell migration and

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The content of this article does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred.

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invasiveness of cultured mammary epithelial cells (MECs) [Xuan et el., 1994; Xuan et al., 1995; Tuck et al., 1999], that it may be secreted in greater quantity by MECs of greater degree of malignancy [Tuck et al., 1999], and that higher levels (tumor cell or plasma levels respectively) are associated with poorer prognosis in patients with either lymph node negative or metastatic breast cancer [Tuck et al., 1998; Singhal et al., 1997]. Evidence from us [Xuan et al., 1994, 1995; Tuck et al., 1999], and others [Senger et al., 1996], has indicated that OPN-induced cell movement is a directed, RGD-dependent response, although CD44-mediated phenomena may also be involved [Weber et al., 1996; Bourguignon et al., 1998, 1999; Katagiri et al., 1999; Tuck et al., unpublished observations]. Cell adhesion studies have shown for a variety of cell types that the major cell surface integrins involved in OPN binding include αvβ1, αvβ3, and αvβ5 [Hu et al., 1995; Liaw et al., 1995].

Given this information, along with the abundant evidence for the importance of HGF/Met in cell motility of MECs [Bhargava et al., 1992; Rosen et al., 1994; Rahimi et al, 1998], and in the malignancy of breast cancer [Yamashita et al., 1994; Tuck et al., 1996; Yao et al., 1996; Jin et al., 1997; Beviglia et al., 1997; Ghoussoub et al., 1998], we set out to examine the nature of OPN-induced cell migration, with respect to the involvement of cell surface integrins known to bind OPN, and possible interactions with the HGF/Met pathway. We have made use of three MEC lines, of differing malignancy: 21PT, non-tumorigenic; 21NT, tumorigenic, non-metastatic [Band et al., 1990]; and MDA-MB-435: tumorigenic, highly metastatic [Price et al., 1990]. We have assessed these cells for migratory responsiveness to OPN, alone and in combination with HGF. Having found evidence for a synergistic relationship between OPN and HGF in inducing cell migration, we proceeded to characterize the cell surface integrins involved, using blocking antibodies to αvβ5, β1, or αvβ3 integrins. OPN-treated cells were then examined in time course experiments for induction of Met kinase activity and tyrosine phosphorylation, and for levels of HGF and Met mRNA and protein. Incubation with OPN was found to result in rapid activation of Met (all three cell lines), followed by an increase in Met RNA (all three cell lines) and protein (21PT and 21NT).

This work thus provides evidence that MEC cell lines representative of different stages of progression make use of different cell surface integrins in the migration response to OPN, and that this OPN-induced cell migration may be mediated at least in part by activation of Met.

METHODS

Cell Lines and Culture

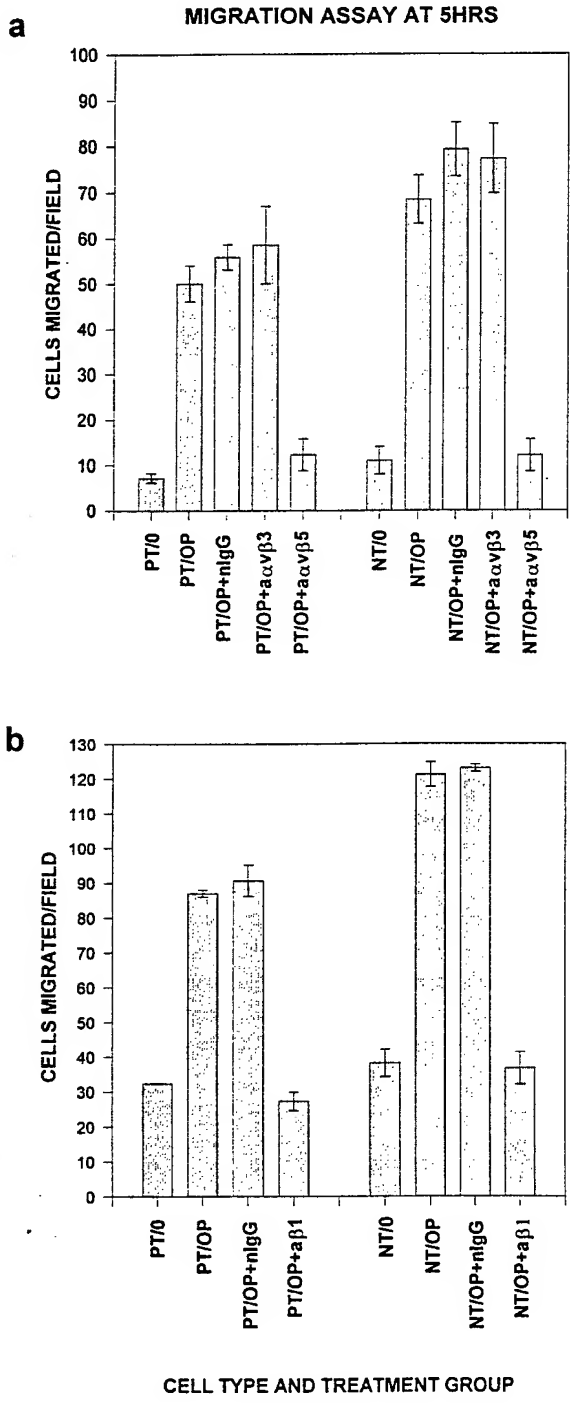
The 21T series cell lines (21PT, 21NT) were obtained as a kind gift of Dr. Vimla Band (Dana Farber Cancer Institute) [Band et al., 1990]. These cells were maintained in culture in α-MEM supplemented with 10% FCS, 2 mM L-glutamine (all from GIBCO-BRL/Life Technologies, Grand Island, NY), insulin (1 μg/ml), epidermal growth factor (EGF; 12.5 ng/ml), hydrocortisone (2.8 μM), 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μg/ml gentamycin (all from Sigma; αHE medium). MDA-MB-435 cells [Price et al., 1990] were obtained as a kind gift of Dr. Janet Price (MD Anderson Cancer Center, Houston, TX), and were grown in α-MEM with 10% FCS (both from GIBCO-BRL/Life Technologies).

Cell Migration

Cell migration assays were performed essentially as described previously [Xuan et al., 1995], using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 6 μg/filter and air dried. The gelatin was rehydrated with 100 μl of serum-free αHE medium at room temperature for 90 min. Lower wells contained 800 μl of αHE plus 0.1% BSA, with or without OPN, HGF, and/or blocking antibodies (as specified in Figs. 1 and 2). Human OPN (50 μg/ml) used was the full length human recombinant GST-OPN (hrOPN), as previously described [Xuan et al., 1994]. Previous control experiments have shown that the GST portion alone has no influence on migration of these cells. Human HGF (20 ng/ml) was obtained from Collaborative Biomedical Products (Becton-Dickinson, Bedford, MA). Blocking anti-integrin antibodies included anti-αvβ3 (Cedarlane, Hornby, ON), anti-αvβ5 (GIBCO-BRL), and anti-β1 (GIBCO-BRL), all used at saturating concentrations as determined by preliminary titration experiments. Cells

(5×10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated for 5 h at 37°C. At the end of the incubation time, the cells that had migrated to the undersurface of the filters were fixed in place with gluteraldehyde and stained with hematoxylin. Cells that had not migrated and were attached to the upper surface of the filters were removed from the filters with a cotton swab. The lower surfaces of the filters were examined microscopically under 100× magnification and representative areas were counted to determine the number of cells that had migrated through the filters. Control experiments were also performed in which blocking antibody in the lower chamber was replaced by non-immune mouse IgG (Cedarlane) at comparable concentration.

All cell migration and invasion assays were performed in triplicate. Statistical differences between groups were assessed using Student's *t*-test, with SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.



Immunoprecipitation and Western Blotting for Met and Phosphotyrosine

Cells in monolayer were grown to 85–90% confluence, serum starved overnight, and incubated in serum-free medium either with or without human OPN (50 μg/ml) or HGF (20 ng/ml) for the times specified. Cells were then rinsed with cold PBS, and lysed in lysis buffer containing 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM PMSF. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. Protein concentration of supernatants was determined using a bicinchoninicacid protein assay (Pierce, Rockford, IL). Equal protein amounts of each lysate were immunoprecipitated with rabbit anti-human Met polyclonal antibody at 4°C for 2 h.

Immunoprecipitates were collected on protein A-Sepharose (Amersham-Pharmacia Bio-

Fig. 1. **a**: OPN-induced migration of 21PT (PT) and 21NT (NT) cells is αvβ5, not αvβ3, integrin-dependent. Migration assays were performed as described in Materials and Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 15 μg/ml non-specific mouse IgG (OP+nIg); 50 μg/ml hrOPN with 30 μg/ml anti-αvβ3 integrin blocking antibody (OP+aαvβ3); or 50 μg/ml hrOPN with 15 μg/ml anti-αvβ5 integrin blocking antibody (OP+aαvβ5). **b**: OPN-induced migration of 21PT (PT) and 21NT (NT) cells is β1 integrin-dependent. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 15 μg/ml non-specific mouse IgG (OP+nIg); or 50 μg/ml hrOPN with 15 μg/ml anti-β1 integrin blocking antibody (OP+aβ1). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

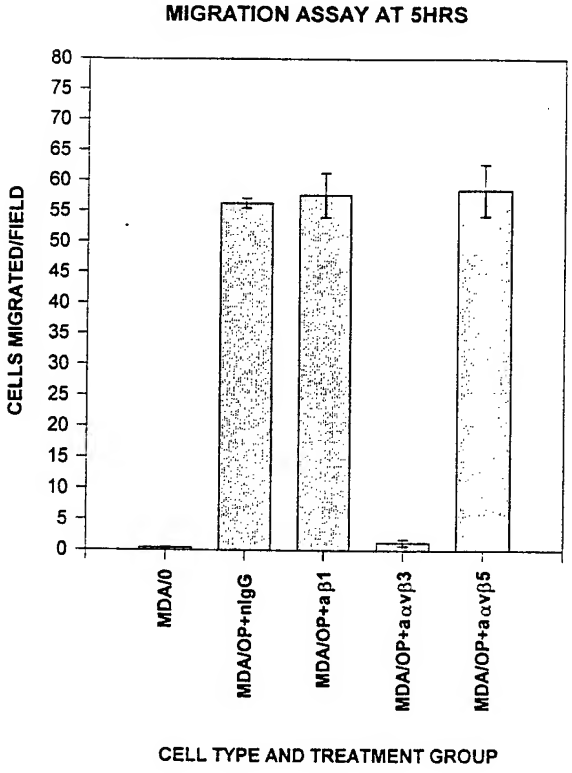


Fig. 2. OPN-induced migration of MDA-MB-435 (MDA) cells is αvβ3, not αvβ5 or β1 integrin-dependent. Migration assays were performed as described in Materials and Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 25 μg/ml non-specific mouse IgG (OP+nIg); 50 μg/ml OPN with 15 μg/ml anti-β1 integrin blocking antibody (OP+aβ1); 50 μg/ml hrOPN with 25 μg/ml anti-αvβ3 integrin blocking antibody (OP+aαvβ3); or 50 μg/ml hrOPN with 15 μg/ml anti-αvβ5 integrin blocking antibody (OP+aαv5). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

tech, Baie d'Urfe, Quebec, Canada), washed three times with lysis buffer, separated by 7% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with 3% skim milk, or 1% BSA, in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and probed for 1 h with either mouse anti-human Met (DL-21 clone, Upstate Biotechnology Inc., Lake Placid, NY) or antiphosphotyrosine antibody (PY20 clone, Transduction Labs, Lexington, KY). The membrane was washed three times for 5 min each with TBST buffer, incubated with horseradish peroxidase-labeled secondary anti-mouse antibody (Amersham-Pharmacia Biotech) for 15 min, and washed three times with TBST for 10 min each. Immune complexes were detected using ECL (Mandel/NEN, Guelph, ON).

In Vitro Met Kinase Assay

Cell cultures incubated in the presence or absence of hrOPN (50 μg/ml) or HGF (20 ng/ml) were rinsed with cold PBS, lysed, and immunoprecipitated as above. Immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4). In vitro Met kinase activity was determined by incubating immunoprecipitates with 20 μl of kinase buffer containing 10 μCi [γ-^{32}P] ATP at 30°C for 10 min. The reaction was stopped by addition of 2× SDS sample buffer containing 5% β-mercaptoethanol. Samples were boiled for 3 min and subjected to 7% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45°C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature and drying for 2 h at 80°C under a vaccum. Autoradiograms were produced and quantitated using a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Analysis of Met and HGF mRNA Levels

Cell cultures (85–90% confluent) were incubated in serum-free medium for the specified times in the presence or absence of 50 μg/ml hrOPN. Cells were harvested by gentle trypsinization, pelleted, and mechanically homogenized (Polytron PT 1200, Brinkman Instruments [Canada] Ltd., Mississauga, ON). RNA was extracted using TRIzol Reagent (Canadian Life Technologies Inc., Burlington, ON), according to the protocol supplied by the manufacturer. RNA (10 μg/lane) was run on a 1.1% agarose gel with 6.8% formaldehyde, and capillary-transferred to GeneScreen Plus filters (DuPont Canada Inc., Mississauga, ON). Blots were probed with denatured, oligolabeled [α^{32}P]-dCTP cDNA probes (labeled using a kit provided by Pharmacia), according to the procedures provided by the manufacturers, and as previously described [Tuck et al., 1990, 1991]. cDNA probes were as follows: hepatocyte growth factor (HGF)—540 bp BamHI-XhoI fragment of human HGF cDNA [Nakamura et al., 1989]; Met/HGF receptor (HGFR)—800 bp EcoRI-EcoRV fragment of the human *met* cDNA [Park et al., 1987]; 18S rRNA (18S)—from p100D9.

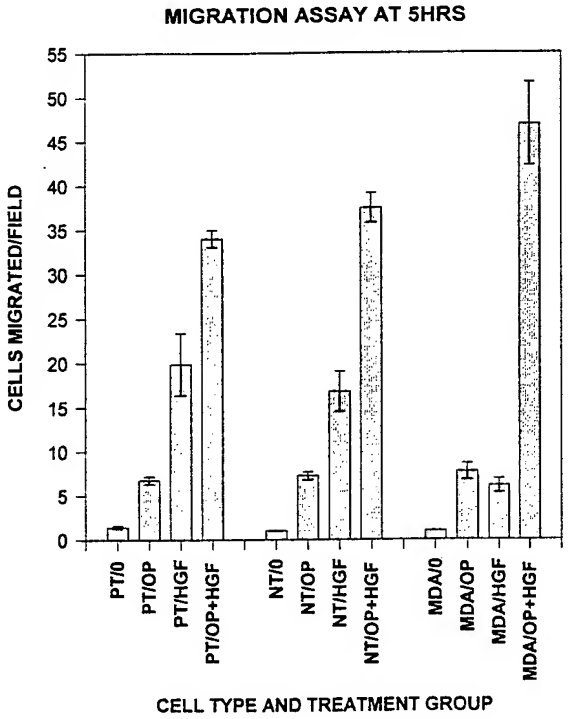


Fig. 3. Synergistic effect of OPN and HGF on migration of 21PT (PT), 21NT (NT), and MDA-MB-435 (MDA) cells. Migration assays were performed as described in Materials and Methods. Contents of the lower chamber consisted of either: medium (αH, no EGF) without HGF or hrOPN (0); medium with 50 μg/ml hrOPN (OP); medium with 10 ng/ml HGF (HGF); or medium with 50 μg/ml hrOPN and 10 ng/ml HGF (OP+HGF). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

RESULTS

OPN-Induced Migration of 21PT and 21NT Cells Involves Different Cell Surface Integrins Than for MDA-MB-435 Cells

Cell migration of 21PT, 21NT, and MDA-MB-435 cells was found to occur in response to hrOPN at a level comparable to that determined previously [Tuck et al., 1999]. Blocking experiments were performed using saturating concentrations of anti-integrin antibodies in the lower chamber of transwells, as described in Methods. For 21PT and 21NT cells, complete blocking of OPN-induced cell migration (to baseline levels) was obtained with the anti-αvβ5 and β1 integrin antibodies (Fig. 1a,b; $P < 0.002$ for all, Student's t-test). In contrast, non-immune mouse IgG did not block migration of either cell line. Saturating concentrations (30 μg/ml) of anti-αvβ3 integrin antibody had no detectable affect on migration of either 21PT or 21NT cells (Fig. 1a).

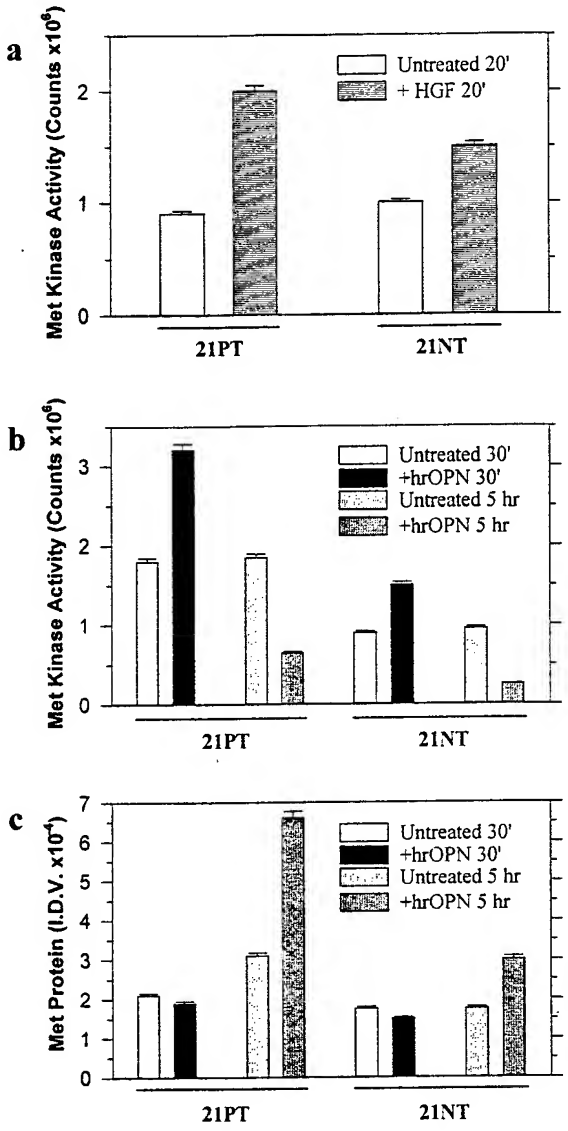


Fig. 4. HGF (a) and OPN (b)-induced increase in total cellular Met kinase activity of 21PT and 21NT cells. OPN (c)-induced increase in total Met protein of 21PT and 21NT cells. a,b: Cells were incubated ±20 ng/ml HGF or 50 μg/ml hrOPN for the times indicated, and cells lysates were prepared. Equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG, and in vitro Met kinase activity was determined as described in Materials and Methods. Quantitation was done using a Phosphorimager. Total Met kinase activity is expressed in cpm/sample. c: Met protein was quantitated by immunoprecipitation with rabbit polyclonal anti-Met antibody, followed by 7% SDS-PAGE and Western blotting as described in Materials and Methods. Total Met protein was quantitated by densitometry and is expressed in integrated density value units. Error bars represent an average standard deviation of 2.5%, as determined by repetitive measurements of individual bands (instrument error). Each graph is representative of at least two separate experiments.

In contrast to results with 21PT and 21NT cells, anti-αvβ5 and β1 integrin antibodies showed no blocking effect on OPN-induced cell migration of MDA-MB-435 cells, when used at the same high concentrations shown to effect complete blocking of 21PT and 21NT responsiveness (15 μg/ml of either anti-integrin antibody; Fig. 2). On the other hand, OPN-induced migration of MDA-MB-435 cells was completely blocked by anti-αvβ3 integrin antibody, at a concentration (25 μg/ml) lower than that which still had no effect on migration of 21PT or 21NT (30 μg/ml; cf. Fig. 1; $P = 0.0008$, Student's t-test).

The OPN-induced migration of the metastatic cell line of this series—MDA-MB-435, thus was found to be αvβ3 integrin-dependent, whereas that of non-metastatic 21NT and 21PT cells was αvβ5 and β1-dependent, αvβ3-independent.

HGF-Induced Cell Migration and Synergistic Effect With OPN

As was found for response to OPN, all three cell lines (21PT, 21NT, MDA-MB-435) showed increased cell migration in response to human recombinant HGF alone (Fig. 3). Combining both HGF and osteopontin in the lower chamber resulted in a degree of cell migration for all three cell lines that was significantly greater than the sum of the isolated HGF and OPN responses (i.e., synergistic; Fig. 3; $P < 0.02$ for all, Student's t-test).

Induction of Met (HGFR) Activity by HGF and OPN

Treatment of 21PT and 21NT cells with either HGF or OPN (Fig. 4a,b) resulted in rapid activation of total Met kinase activity in both instances (after 20 min of HGF stimulation, 30 min of OPN stimulation). For both 21PT and 21NT cells, the increase in total Met kinase activity with OPN treatment (at 30 min) was followed by an increase in Met protein level at 5 h of incubation with OPN (Fig. 4c).

Treatment of MDA-MB-435 cells with HGF or OPN (Fig. 5a) resulted in an increase in specific Met kinase activity after 20 (for HGF) to 30 (for OPN) min of incubation, which was associated with an increased tyrosine phosphorylation of Met as well (Fig. 5b). In contrast with 21PT and 21NT cells, we have not been able to detect an OPN-induced increase in total

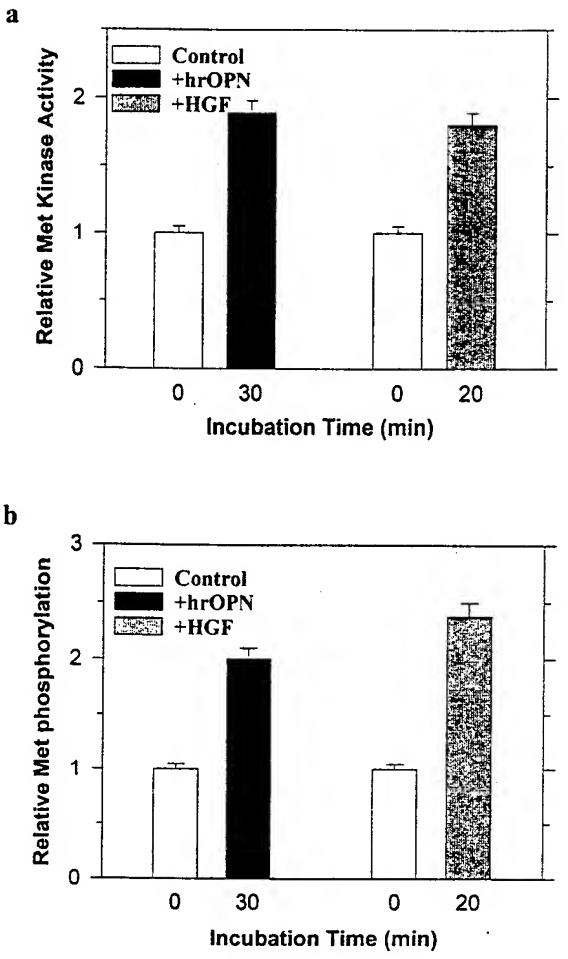


Fig. 5. Induction of specific Met kinase activity (a) and Met tyrosine phosphorlyation (b) of MDA-MB-435 cells by HGF and OPN. MDA-MB-435 cells were incubated ±50 μg/ml hrOPN or 20 ng/ml HGF for the times indicated. Cell lysates were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG. As levels of total Met protein were higher and fluctuated more in MDA-MB-435 cells than in 21PT and 21NT, activation of Met protein in MDA-MB-435 was more appropriately expressed as Relative Met kinase activity (a) and tyrosine phosphorylation (b). a: In vitro Met kinase activity was assayed as described in Materials and Methods. Relative Met kinase activity, normalized to total Met protein, was quantitated using a Phosphorimager. b: Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with 1% BSA in TBST, and probed with anti-phosphotyrosine antibody. Detection was performed with HRP-labeled anti-mouse antibody and ECL. Relative Met tyrosine-phosphorylation normalized to total Met protein was quantitated by densitometry. Error bars represent an average standard deviation of 5.0%, as determined by repetitive measurements of individual bands (instrument error). Each graph is representative of at least two separate experiments.

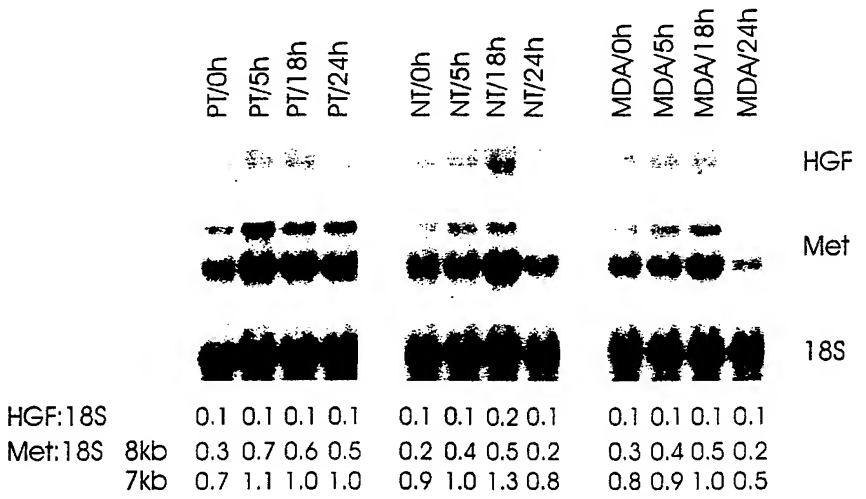


Fig. 6. Time course showing effect of OPN on expression of HGF and Met mRNA by 21PT (PT), 21NT (NT), and MDA-MB-435 (MDA) cells. Near-confluent (85–90%) cell cultures were incubated in serum-free medium with 50 μg/ml OPN for 0, 5, 18, or 24 h. Total RNA (10 μg/lane) was analysed by Northern blotting for expression of HGF (6.0 kb) or Met (HGFR; 8.0 kb [full-length transcript, upper band], 7.0 kb [lower band]). RNA loading and integrity were verified by assessment of 18S rRNA (2.1 kb). Level of HGF and Met (both 8kb and 7kb transcripts) are shown in relation to 18S rRNA, expressed as the ratio of densitometry values for the respective bands (HGF:18S, Met:18S).

Met protein levels in MDA-MB-435 cells, although basal level of Met protein expression in MDA-MB-435 is higher than in 21PT or 21NT (data not shown).

Time Course Showing Effect of OPN on Expression of HGF and Met mRNA

21PT, 21NT, and MDA-MB-435 cells treated with OPN (50 μg/ml) for 0–24 h (Fig. 6), all showed low basal levels of HGF mRNA. Only slight increase in HGF mRNA was detected for 21PT and 21NT, with no appreciable increase for MDA-MB-435 cells (by 18–24 h). In contrast, levels of Met RNA were found to significantly increase in all three cell lines between 5 and 18 h of OPN exposure, falling off by 24 h. Thus, although little change in HGF mRNA was seen after up to 24 h of OPN exposure, significant induction of Met mRNA was seen for all three cell lines.

DISCUSSION

OPN has been implicated in the maligancy of breast cancer in a number of recent studies [e.g., Oates et al., 1996; Singhal et al., 1997; Sung et al., 1998; Tuck et al., 1998, 1999]. It has been shown to be involved in cell adhesion of MECs, and can also induce cell migration in an RGD-dependent fashion [Xuan et al., 1994, 1995; Senger et al., 1996]. The HGF/Met pathway has also been associated with breast cancer malignancy [Yamashita et al., 1994; Tuck et al., 1996; Yao et al., 1996; Jin et al., 1997; Beviglia et al., 1997; Ghoussoub et al., 1998], and is a potent inducer of MEC motility [Bhargava et al., 1992; Rosen et al., 1994; Rahimi et al., 1998]. Here we examine the nature of the integrin response to OPN, in order to establish the specific cell surface integrins involved. We also show that OPN-induced cell migration involves activation of the HGF receptor in a syngergistic fashion with HGF, consistent with cross-talk between integrin and growth factor mediated pathways.

Our discovery that, in a series of breast epithelial cells of differing degrees of malignancy, different cell surface integrins may couple with Met in inducing cell migration is a novel finding. The metastatic member of the series studied, MDA-MB-435 cells, showed the most marked synergy between OPN and HGF in the migration response, and migrated in an αvβ3, not αvβ5 or β1-dependent fashion. In contrast, the non-metastatic cell lines, 21PT and 21NT, migrated in an αvβ5 and β1 dependent, αvβ3-independent fashion. In support of this finding is the work of Wong et al. [1998], who reported that MDA-MB-435 cells express αvβ3 integrin, while less maligant MDA-MB-231 and MCF-7 cells do not (although they all express αvβ5 and

β1). Similarly, van der Pluijm et al. [1997] reported higher expression of αvβ3 in more malignant members of a series of breast carcinoma cell lines. A specific association between αvβ3 expression and breast cancer metastasis has also been reported by Liapis et al. [1996], who detected αvβ3 integrin expression in 100% of breast carcinomas that had metastasized to bone.

This difference in integrin utilization of cells at different stages of progression could affect malignancy in a number of different ways. For example, αvβ3 may be necessary for specific adhesion events vital to invasion and metastasis; a breast cancer cell initially expressing αvβ1 or αvβ5 may require activation of β3 in order to complete that step of the metastatic cascade. Evidence in favor of this scenario (at least in the case of melanoma) comes from the work of Nip et al. [1992], who showed that binding of metastatic cells to lymph node matrix depends on αvβ3 interactions. Alternatively, different integrins may be coupled to different signal transduction pathways, with αvβ3 specifically required for activation of a particular set of genes important in aspects of invasion and metastasis. Ligation of αvβ3 for example, has been shown to induce MMP-2 expression and invasion of melanoma cells [Seftor et al., 1992; Bafetti et al., 1998]. Whether or not different integrins couple to Shc can influence activation of transcription from the Fos serum response element (SRE), affecting responsiveness to growth factors [Wary et al., 1996]. In the case of coupling with the HGF/Met pathway, our work suggests that OPN-induced cell migration via either non-αvβ3 integrins (21PT, 21NT) or αvβ3 integrin (MDA-MB-435) is associated with Met activation, but that the synergistic effect on cell migration is much more pronounced in the cells (MDA-MB-435) expressing αvβ3.

We also found that OPN-induced migration of all three cell lines involves activation of the HGF receptor, Met, with an initial increase in Met activity followed by an increase in Met RNA expression. The kinetics of this effect differ slightly for MDA-MB-435 vs. the 21T series cells. For 21PT and 21NT, a detectable increase in Met protein was also found. Although a similar increase in Met protein levels of MDA-MB-435 cells did not occur, the basal level of Met protein in these cells is quite high, and as specific activity of Met is substantially increased with OPN induction, it is possible that Met turnover is such that protein levels do not further accumulate as they do for the 21T series cells. Regardless, Met is activated by OPN in all three cell lines, and this is associated with increased cell migration. In contrast, HGF mRNA levels were low in all three cell lines, and showed little or no change with OPN treatment. Furthermore, we have not detected increased HGF activity in conditioned media of OPN-treated 21T series or MDA-MB-435 cells (data not shown). Activation of Met by OPN is thus likely due to either an increased sensitivity to trace amounts of ligand present, or to ligand-independent activation. Ligand-independent activation of Met by cellular adhesion has been previously reported for melanoma cells, although the cell surface adhesion receptors involved were not examined [Wang et al., 1996]. Furthermore, integrin binding has been shown to be essential for growth factor (EGF, PDGF, bFGF, IGF-1) induced signal transduction and cell migration [Miyamoto et al., 1996; Brooks et al., 1997]. Reciprocally, HGF can activate cell surface integrins and hence cellular adhesion (and motility) [van der Voort et al., 1997; Weimar et al., 1997, 1998; Trusolino et al., 1998]. Thus, a two-way interaction between integrin and growth factor- mediated pathways likely occurs in the induction of cellular responses such as cell migration.

Multiple points of interaction between signal transduction pathways activated by integrins vs. growth factors have been identified [reviewed in Sastry and Horowitz, 1996; Giancotti, 1997; Swartz, 1997]. Cell attachment can enhance autophosphorylation of growth factor receptors (EGFR, PDGFR, and now Met) in response to their cognate ligands. Integrin binding also has been found to activate phospholipase C (and hence protein kinase C), Raf, and/or MEK in the MAP kinase pathway, and PI-3 kinase in the PI-3K/Rac pathway. All of these pathways are also influenced by growth factors, although the synergistic relationship reported here would suggest that growth factor receptors and integrins may act at different points in the pathway. For example, it has been shown that fibronectin binding to cell surface integrin activates synthesis and supply of phosphatidylinositol 4,5 biphosphate, whereas PDGF receptor controls the activity of phospho-

lipase C [McNamee et al., 1992]. The physical association of integrins and growth factor receptors at the focal adhesion complex (FAC) [Plopper et al., 1995] provides a mechanism by which such cross-talk would be facilitated. Finally, interactions between growth factor and integrin pathways could occur at the level of differential effects on members of the FAC itself [Schlaepfer and Hunter, 1998].

In the case of signal transduction initiated by OPN, ligation of αvβ3 by OPN activates PI-3 kinase in osteoclasts [Hruska et al., 1995]. In an osteoblastic cell line (UMR 106-6), OPN triggers the autophosphorylation of focal adhesion kinase (FAK) [Liu et al., 1997]. In *ras*-transformed fibroblasts, OPN can induce tyrosine phosphorylation of a number of different FAC associated proteins [Lopez et al., 1995]. HGF activation of its receptor, Met, can also stimulate phosphorylation of FAK in some cells, perhaps via $pp60^{src}$ [Chen et al., 1998]. We have shown in this report that OPN is also capable of activating Met. Thus, although our understanding of the signal transduction pathways induced by OPN is yet in early stages, multiple points of potential interaction between the integrin and growth factor receptor pathways involved are already beginning to emerge.

The interactions between different integrin pathways induced by OPN and the HGF/Met growth factor pathway not only helps conceptually in understanding the clinical associations we have observed between OPN, HGF and malignancy, but also provides clues to regulatory processes vital to tumor aggressiveness—prime targets for treatment strategies based on blocking these processes.

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Appendix IV

Regulation of expression of hepatocyte growth factor in mammary carcinoma cells via a c-Src tyrosine kinase/Stat3 pathway

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Running title: A c-Src/Stat3 pathway activates HGF expression

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(Submitted)

Abstract

Over-expression of HGF and its receptor, Met, occurs in many types of cancer including breast. We have previously shown co-expression of HGF and Met in the invasive tumor front of human breast carcinomas, and secretion of HGF and constitutive activation of Met and downstream signalling molecules in the murine breast carcinoma cell line, SP1. These observations suggest the presence of an HGF autocrine loop in breast carcinoma cells, which confers survival and growth advantage to carcinoma cells during tumor progression and metastasis. c-Src tyrosine kinase, which is critical in regulating the expression of many genes, is activated in SP1 cells. In this study we examined the role of c-Src in HGF expression in breast carcinoma cells. We found that *HGF* mRNA and protein levels changed accordingly to the level of c-Src kinase activity in SP1 cells. Expression of activated c-Src increased transcription from the *HGF* promoter while dominant negative c-Src had the opposite effect. Using deletion analysis, we showed that the region between -254 and –70 bp was required for c-Src responsiveness of the *HGF* promoter. This region contains two putative consensus sequences for Stat3 (at –110 and –149 bp) which bind Stat3-containing protein complexes. Co-expression of activated c-Src and Stat3 synergistically induced *HGF* promoter activity. c-Src kinase activity correspondingly changed the tyrosine phosphorylation and DNA binding affinity of Stat3 (but not Stat1, -5A or -5B). Collectively, our data indicate that c-Src kinase regulates *HGF* transcription and protein expression in mammary carcinoma cells through activation of Stat3, and thereby promotes a tumorigenic phenotype.

Introduction

Scatter factor, also known as hepatocyte growth factor (HGF), is a multi-functional cytokine. Through binding to its receptor (Met), HGF can induce cell survival (72,93), growth (77), differentiation (63) and motility (30). It has been shown that both HGF and Met are essential for embryo development. Disruption of HGF expression in mice results in lethality in early development (79), while deletion of Met causes underdevelopment of limb buds (2,95). During development of the mammary gland, HGF is expressed by stromal cells, whereas epithelial cells express Met, but not HGF (2,97). Paracrine stimulation of normal breast epithelium with HGF, in co-operation with other growth factors (e.g., neuregulin), promotes branching morphogenesis (9). The tissue specific suppression of HGF in normal epithelial cells provides a tightly controlled regulation of mammary ductal morphogenesis (11).

In contrast to normal breast epithelium, HGF and Met are frequently over-expressed in breast carcinomas as well as many other cancer types (19-23,27,31). This high level of HGF and Met expression has been identified as a possible independent predictor of poor survival in breast cancer patients (94). Our laboratory has previously shown that invasive human carcinoma cells co-express HGF and Met, particularly at the migrating tumor front (87). We have also found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met but not HGF (Gui *et al.*, unpublished results). Furthermore, over-expression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (52,86) or in transformed cell lines (6,29,39) promotes tumorigenesis and metastasis. Together, these results suggest that establishment of an autocrine HGF loop and sustained activation of the Met signal transduction pathway in carcinoma cells may promote tumor progression. However, the mechanisms

leading to aberrant expression of HGF in carcinoma cells are not known.

A number of signalling molecules, such as c-Src (74), Ras (35), Grb2 (28) and phosphatidylinositol (PI) 3-kinase (12,72), have been shown to be part of the HGF/Met signalling pathway. Activation of Met through binding of HGF causes auto-phosphorylation of two specific tyrosine residues in the cytoplasmic tail of the receptor tyrosine kinase (70,71). These phosphorylated tyrosine residues act as multi-functional docking sites that bind the SH2-domain of specific cytoplasmic signalling molecules and causes their activation. The c-Src non-receptor tyrosine kinase is expressed in many cell types, and its activity is increased in response to HGF and binding to Met (74). Increased activation of the tyrosine kinase c-Src occurs in many human cancer cells, and c-Src plays a critical role in breast cancer. Over-expression of an activated form of c-Src in transgenic mice induces mammary hyperplasia (90). Furthermore, c-Src kinase is required in polyoma middle T-induced mammary tumorigenesis in transgenic mice (33). We have shown previously that c-Src kinase is constitutively activated in a mouse breast carcinoma cell line, SP1, which expresses both HGF and tyrosine-phosphorylated Met and which exhibits spontaneous invasion through matrigel (25,72,74-76,78). Furthermore, c-Src kinase activity is required for HGF-dependent cell motility and anchorage-independent growth of SP1 cells (74). Collectively, these findings indicate that c-Src kinase is an important requirement, but is not sufficient, for mammary tumorigenesis.

Activation of c-Src kinase can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (VEGF) (64,65) and parathyroid hormone-related peptide(44). We therefore hypothesized that elevated c-Src activity can promote increased HGF expression and the establishment of an HGF autocrine loop in SP1 cells. We observed that the Src tyrosine kinase inhibitor PP2 causes a two-fold reduction in *HGF* transcription in SP1 cells. In

addition, expression of a dominant negative mutant of c-Src (SRC-RF) in SP1 cells leads to similar levels of reduction in *HGF* mRNA and functional protein. Using deletion mutants of the *HGF* promoter, we have located a region (between –254 and –70) of the *HGF* promoter responsive to increased c-Src kinase activity in SP1 cells. This region contains two putative consensus binding sites for Stat3. Stat3 is a transcription factor originally described as the target of interferon receptors (50,57), but recent reports have indicated that Stat3 can be activated by c-Src kinase via PDGF (89) and HGF receptors (11), and is important in mammary differentiation (11). We therefore examined the role of Stat3 in c-Src-dependent regulation of *HGF* transcription. The results indicate that while expression of Stat3 alone increased *HGF* promoter activity, simultaneous expression of Stat3 and activated c-Src led to strong cooperative activation of *HGF* transcription. Expression of mutant c-Src kinases in breast carcinoma cells altered both the tyrosine phosphorylation status and DNA binding activity of Stat3. While activated c-Src can induce Stat3 tyrosine phosphorylation and DNA binding activity, a dominant negative mutant of c-Src reduced tyrosine phosphorylation and DNA binding. Together these data suggest that c-Src kinase activity is important in regulating HGF expression in breast carcinoma cells, and that the mechanism of regulation is through the transcription factor Stat3.

Materials and Methods

Antibodies and reagents: Rabbit anti-c-Src IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody EC10 against chicken c-Src was a gift from Dr. S. Parsons. Rabbit anti-sheep IgG conjugated with horseradish peroxidase was from Jackson ImmunoResearch

Laboratories (West Grove, PA). Sheep anti-HGF IgG was a gift from Genentech (San Francisco, CA). Rabbit anti-HGF antibody was generated against recombinant GST-HGF (1-120) protein in our laboratory at Queen's University, this antibody recognizes only the N-terminal portion of HGF (data not shown). Anti-Stat1, -Stat3, -Stat5A and -Stat5B and anti-phospho-Stat3 (Y705) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). c-Src family kinase inhibitor PP2 was obtained from Calbiochem (LaJolla, CA).

Plasmid Construction: c-Src expression plasmids were constructed by subcloning activated (Y527F) and dominant negative (K295F, Y527F) chicken *c-src* cDNAs (gift from Drs. J. Brugge and D. Shalloway) into the EcoRI site of DNA polymerase I (Klenow fragment)-treated pBabePuro plasmid to generate pBabe 527 and pBabe Src RF. A reporter construct containing the full length *HGF* promoter region fused to luciferase (2.7 HGF-luc) was constructed by ligating the HinD III/ Xba I fragment (treated with DNA polymerase I (Klenow fragment)) of 2.8 HGF-CAT (gift from Dr. R. Zarnegar) into the HinD III site of pGL2-Basic (Promega), also treated with DNA polymerase I (Klenow fragment). Further deletions were constructed by cutting 2.7 HGF-luc with Sma I, Sac I and Bgl II, followed by re-ligation to generate 0.5 HGF-luc, 0.3 HGF-luc and 0.1 HGF-luc, respectively. The 1.2 HGF-luc was constructed by ligating the 1.4 kb Sal I fragment from 2.7 HGF-luc into the Xho I site of pGL2-Basic. An internal deletion mutant 0.5Δ HGF-luc was constructed by digestion of 0.5 HGF-luc with Pvu II/ Bgl II and treatment with DNA polymerase I (Klenow fragment) before re-ligation. The Δ1 HGF-luc was constructed by ligating the Sma I fragment of 2.7 HGF-luc into the same site of 0.5Δ HGF-luc. The Δ2 HGF-luc was constructed by ligating the Sma I fragment of 2.7 HGF-luc into 0.8 HGF-luc. The ΔΔ HGF-luc was made by ligating the Sma I fragment of Δ2 HGF-luc into the same site of 0.5Δ HGF-luc. For normalization of transfection

effeciency of each sample, pSG5βgal (a gift from Dr. M. Petkovich) or pCHCβgal (a gift from Dr. F. Kern) (58), which expresses β-galactosidase under the control of SV40 and CMV promoters, respectively, was used.

Tissue Culture and Cell Lines: The SP1 tumor cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma, it expresses HGF and Met. The characterization of the SP1 cell line has been described previously (25,76,78). Maintenance medium for SP1 cells was RPMI 1640 supplemented with 7% fetal bovine serum. Cos-1 cells were obtained from ATCC and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. HC11 cells are a mammary epithelial cell line (24) and were maintained in RPMI 1640 supplemented with 10% FBS, insulin (5 μg/ml) and EGF (10 ng/ml).

Cell Transfection: All transfections were carried out with Lipofectamine Plus reagent (Canadian Life Technology, Burlington, ON) according to manufacturer's instructions. Cells (15,000) were seeded in a 24-well plate and transfected with 0.4 μg of reporter plasmid, 0.1 μg pSG5βgal and up to 0.4 μg of expression plasmids (such as c-Src) as indicated. 48 hours post-transfection, cells were harvested and lysed. One-fifth of the lysate was used to assay for β-galactosidase activity, an equal amount of lysate was used for luciferase assay using PharMingen Luciferase Substrates (BD PharMingen, Mississauga, ON). Luciferase activity was measured in a luminometer at 562 nm. Luciferase activity of each sample was normalized to the corresponding β-galactosidase activity. For immunoprecipitation and *in vitro* c-Src kinase assays, 2.5 x 10^5 cells were seeded on a 100 mm tissue culture plate and transfected with 4 μg of reporter plasmid, 1 μg pSG5-βgal and up to 4 μg of expression plasmids as indicated. One tenth of the cells was saved for luciferase assay and the rest was lysed and used for immunoprecipitation.

To obtain stably transfected cells, SP1 cells were plated at 70% confluence in 60 mm plates and transfected with 2 μg of plasmids expressing various mutants of c-Src. Puromycin (2 μg/ml, Sigma, Oakville, ON) was added to cells 24 hours post-transfection and was maintained until all cells in mock transfection were killed. Puromycin resistant cells were then collected and used as pooled. Expression and activity of c-Src mutant were checked with western blotting analysis and c-Src kinase assay.

RNA Isolation and RT-PCR: Cells grown to 80% confluence on a 100 mm dish were washed and lysed with TriZol reagent (Canadian Life Technology). Phase separation was achieved by addition of chloroform and centrifugation at top speed in a micro-centrifuge for 10 minutes. Aqueous phase containing total RNA was removed to a new tube and precipitated with an equal volume of isopropanol for 10 minutes at room temperature. The RNA pellet was recovered by centrifugation and washed with 70% ethanol. After brief drying, the RNA pellet was resuspended in DEPC-treated water. RNA concentration was determined by spectrophotometry. 1 μg of total RNA was used for reverse transcription with AMV reverse transcriptase at 42°C for 15 minutes. One tenth of the reaction was used in PCR analysis with end-labelled oligonucleotides specific for HGF (5'-TGTCGCCATCCCCTATGCAG-3' and 5'-GGAGTCACAAGTCTTCAACT-3') and β-glucuronidase (GUS B) sequence as previously described (38,61). PCR reaction conditions were 2 minutes at 95°C, followed by 25 cycles of 1 minute at 95°C, 1 minute at 55°C, 1 minute at 72°C and a final cycle of 10 minutes at 72°C. The reaction was then analyzed on a 2% agarose gel by electrophoresis. The bands corresponding to the HGF and GUS B products were excised and the amount of radioactivity was determined by scintillation counting technique.

Copper affinity column chromatography: Conditioned media were collected and analysed by copper (II) affinity column chromatography as described previously (73). Cells were grown to 80% confluence. The cell monolayer was washed with fresh DMEM and incubated in serum-free DMEM for 24 h. The conditioned media was collected and cells were removed by centrifugation. The conditioned media was then loaded onto a copper (II) affinity column. The copper (II) affinity column was prepared by chelating Cu^{+2} ions on a 1 ml HiTrap Chelating column (Amersham Pharmacia Biotech, Baie d'Urfe, PQ) and equilibrated with equilibration buffer (20 mM sodium phosphate, pH 7.2, 1 M NaCl, 1 mM imidazole). The conditioned media was recycled through the column for 5 times to ensure binding of all HGF proteins, and the column was washed thoroughly with 15 volumes of equilibration buffer. HGF protein was eluted from the column with equilibration buffer containing 80 mM imidazole at a flow rate of 1 ml/min. Fractions of 1 ml each were collected, previous experiments have determined that all HGF was eluted in fraction#2 (data not shown). The fraction containing HGF was concentrated by centrifugation with Microcon centrifugal filter devices (Millipore Corporation, Bedford, MA) with a 10 kDa molecular weight cut off. The samples were analyzed on a denaturing SDS PAGE gel, followed by western blotting analysis with anti-HGF antibody.

Immunoprecipitation, western blotting analysis and c-Src kinase assay: Cells were grown to confluence and treated as indicated. After three washes with cold phosphate-buffered saline, cells were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1 mM PMSF. Cell debris was removed by centrifugation and protein concentrations were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). For immunoprecipitation, equal amounts of

lysate were incubated with the indicated antibodies at 4°C for 2 h or overnight. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, separated by SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blotting analysis was performed as described previously (74).

In vitro c-Src kinase assays were performed as described previously (74). Briefly, each lysate was inmmunoprecipitated with anti-c-Src IgG (Santa Cruz Biotechnology) as described above. One half of each immunoprecipitate was subject to SDS-PAGE under non-denaturing conditions and Western blot analysis to confirm the amount of c-Src protein present. The other half of each immunoprecipitate was assayed for c-Src kinase activity by incubating with 10 µl reaction buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 µM Na_3VO_4), 1.4 µg of freshly prepared acid-denatured enolase (Sigma) and 10 µCi of [γ-^{32}P]-ATP. After 10 min incubation at 30°C, reactions were terminated by the addition of 2X SDS sample buffer, and samples were subjected to 8% SDS PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45°C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature. The gel was dried under vacuum. Autoradiograms were produced and analyzed with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Oligonucleotides and Probe labelling: Oligonucleotides used for EMSA binding were Stat3-110F (5'-GGGCTGTTGTTAAACAGT-3'), Stat3-110R (5'-AGAACTGTTTAACAACAG-3'), Stat3-149F (5'-GGGGTTGAGGAAAGGAAG-3'), and Stat3-149R (5'-CCCCTTCCTTTCCTCAAC-3'). Complementary oligonucleotides were annealed by boiling equal molar amounts of each oligonucleotide for 10 minutes and then cooling slowly to room temperature. 20 pmol of annealed oligonucleotides were labelled by filling-in reaction with Klenow enzyme and [α-^{32}P] dCTP.

Preparation of nuclear extracts and electrophoretic mobility shift assay: Nuclear extracts were prepared as described previously (3). Briefly, 10^7 cells were washed once with PBS before resuspension in cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.5 mM sodium orthovanadate). Cells were allowed to swell on ice for 10 minutes before lysis by brief vortexing. Nuclei were pelleted and resuspended in buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.2 mM PMSF, 0.5 mM sodium orthovanadate). High salt extraction was performed by incubation on ice for 30 minutes in buffer C and centrifugation at 4°C. The protein content of the supernatant (nuclear extract) was determined by Bradford protein assay (BioRad, Mississauga, ON).

Electrophoretic mobility shift assay (EMSA) was performed as described by Mohan *et al* (62). Briefly the binding reaction was performed by incubating 5 μg of nuclear extracts with 0.1 pmol of ^{32}P-labelled oligonucleotide probe in the presence of binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 1 mM DTT), 9% glycerol and 4 μg poly (dI-dC) (Amersham-Pharmacia Biotech). Binding was allowed to proceed at room temperature for 10 minutes before analysis on 5% non-denaturing PAGE gel in Tris-glycine buffer (40 mM Tris-HCl, pH 8.4, 266 mM glycine). When unlabelled oligonucleotides were added, 10-fold molar excess was included in the binding reaction. For supershifting experiments, nuclear extracts were incubated with 2 μg of the indicated antibody at room temperature for 20 minutes prior to the binding reaction. After electrophoresis, the gel was fixed in 7% acetic acid, 40% methanol for 30 minutes, and dried under vacuum. The gel was then exposed to a PhosphorImager screen and analyzed using the Storm PhosphorImager.

Results

Inhibition of activity of c-Src family kinases impairs *HGF* mRNA expression:

To study the regulation of HGF expression in breast carcinoma cells, we used the mouse mammary carcinoma cell line SP1, which co-expresses HGF and tyrosine-phosphorylated Met (25,76). Semi-quantitative RT-PCR was performed to determine the levels of *HGF* mRNA in SP1 cells. We first examined the dose-dependent effect of an inhibitor of c-Src family kinases, PP2 (34). Total RNA was isolated from SP1 cells treated with different concentrations of PP2 and used for cDNA synthesis by reverse transcription. Relative *HGF* mRNA levels were determined by RT-PCR with HGF-specific primers, and each sample was normalized to the expression of a house keeping gene β-glucuronidase (GUS B) (38,61). The results showed that the PP2 inhibitor reduced *HGF* mRNA expression in a dose-dependent manner up to 40% of untreated cells (Fig. 1A). In addition, we examined the level of transcription of the *HGF* gene using a reporter plasmid. A plasmid containing a 2.7 kb fragment 5' of the *HGF* transcriptional start site ligated to the firefly luciferase gene was transiently transfected into SP1 cells. Bell *et al.* (5) have previously shown that this 2.7 kb fragment of the *HGF* promoter contains all the necessary sequence to direct HGF expression and mimics the expression pattern of endogenous *HGF* gene in transgenic mice. Following transfection, these cells were treated with different concentrations of the PP2 inhibitor under conditions used in Fig. 1A. After a 24 h incubation, the cells were lysed and luciferase activity in each sample was determined and compared to control cells. The results show a similar dose-dependent reduction of *HGF* transcription following PP2 treatment (Fig. 1B). These findings suggest that the activity of c-Src kinase family members is important in the regulation of *HGF* mRNA expression.

c-Src kinase activity regulates HGF expression at both mRNA and protein levels:

We further investigated the role of c-Src tyrosine kinase in HGF expression by transfecting chicken c-Src mutants (SRC-Y527F and SRC-RF) with altered kinase activity into SP1 cells. The SRC-Y527F mutant contains a phenylalanine substitution at tyrosine 527 which results in constitutive kinase activity (15,84,85). The SRC-RF mutant contains a double substitution at tyrosine 527 to phenylalanine and at lysine295 to arginine, which produces a dominant negative phenotype (65). We have previously shown that expression of a similar dominant negative form of murine c-Src in SP1 cells reduces endogenous c-Src kinase activity and also impairs anchorage-independent growth in soft agar (74). As predicted, expression of the dominant negative form of chicken c-Src (SRC-RF) also decreased total c-Src kinase activity in SP1 cells, when compared to untransfected cells (Fig. 2, top panel). In addition, expression of the activated form of c-Src (SRC-Y527F) dramatically increased total c-Src kinase activity in SP1 cells. Expression of the chicken c-Src mutants was detected by an antibody (EC10) specific for avian c-Src (Fig. 2, bottom panel).

To assess the effect of c-Src kinase activity on *HGF* mRNA expression, RT-PCR analysis was carried out on RNA extracted from SP1 cells expressing the different c-Src mutants, or treated with the PP2 inhibitor (Fig. 3A). Expression of the dominant negative SRC-RF mutant or treatment with PP2 reduced the *HGF* mRNA level in SP1 cells by approximately 60%. Conversely, expression of the constitutively active SRC-Y527F mutant increased *HGF* mRNA expression by about two-fold. In a parallel approach, the level of secreted HGF protein was compared in conditioned media collected from the same cells and under the same conditions described in Fig. 3A. Our laboratory has previously shown that HGF is a Cu (II) binding protein, which can be purified from conditioned media with copper (II) affinity chromatography (73) and analyzed on a denaturing SDS-PAGE gel

(Fig. 3B). Using this method, we showed that expression of the dominant negative SRC-RF mutant or treatment with PP2 significantly decreased the amount of HGF protein secreted by SP1 cells. In contrast, expression of activated c-Src (SRC-Y527F) increased the amount of secreted HGF protein. Together these data suggest that HGF expression (both at the mRNA and protein levels) is regulated by c-Src kinase activity.

c-Src kinase activity induces HGF expression through a specific *cis*-acting region on the *HGF* promoter:

To facilitate the mapping of the c-Src kinase responsive region(s) on the *HGF* promoter, we constructed a series of reporter plasmids with the luciferase gene linked to different fragments of the 2.7 kb region 5' of the *HGF* transcriptional start site (Fig. 4B). These reporter constructs were co-transfected into SP1 cells with a control vector, or vectors expressing the SRC-Y527F or SRC-RF mutants of chicken c-Src kinase, and luciferase activity of the transfected cells was compared (Fig. 4A). The results show that deletion of up to -1231 bp (1.2 HGF-luc) of the *HGF* promoter did not affect promoter activity in response to the activated c-Src kinase mutant, compared to the activity of the -2.7 kb full length *HGF* promoter-luciferase construct. A further deletion (0.8 HGF-luc) between -1231 and -755 bp identified a small (20%) reduction in c-Src kinase responsiveness of the *HGF* promoter, compared to the full length (-2.7 HGF-luc) or -1.2 kb (1.2 HGF-luc) *HGF* promoter constructs, this finding suggests a possible *cis*-acting element located within this region. Deletion to either -538 (0.5 HGF-luc) or -273 bp (0.3 HGF-luc) did not further change the inducibility of the *HGF* promoter compared to the 0.8 HGF-luc construct. The remaining c-Src kinase responsiveness was eliminated when all but 72 bp (0.1 HGF-luc) of *HGF* promoter was removed. This suggests that

another *cis*-acting element responsive to c-Src kinase activity is located within -273 and -70 bp of the *HGF* promoter. An internal deletion construct removing -70 to -254 (named 0.5Δ HGF-luc) was used to confirm the c-Src responsiveness of this region. As predicted, the 0.5Δ HGF-luc reporter did not respond to expression of SRC-Y527F. A similar pattern of repression of the luciferase activity among all the *HGF* promoter deletion mutants used was seen when dominant negative c-Src (SRC-RF) was co-expressed with the HGF-luc constructs.

To confirm the importance of the regions of the promoter responsive to activated c-Src, several internal deletion mutants were constructed. Full length reporter constructs missing -273 to -70 bp (Δ1), -1231 to -755 bp (Δ2), or both regions (ΔΔ) of the *HGF* promoter were transfected into SP1 cells in the presence or absence of the SRC-Y527F and SRC-RF mutants (Fig. 4A). As predicted, Δ1 and ΔΔ deletion mutants exhibited neither induction nor repression of *HGF* promoter activity when activated c-Src or dominant negative c-Src was expressed, respectively. In contrast, the Δ2 mutant showed strong induction of *HGF* promoter activity corresponding to expression of the activated SRC-Y527F mutant, and strong repression of *HGF* promoter activity when SRC-RF mutant was expressed. This finding shows that only the region between -273 and -70 bp of the *HGF* promoter is important for c-Src responsiveness of HGF expression in SP1 cells. We will refer to this region as the c-Src responsive region.

Stat3 activates *HGF* promoter in co-operation with activated c-Src:

Examination of the c-Src responsive region of the *HGF* promoter revealed several Stat3 binding sites. This consensus sequence is highly conserved among mouse, rat and human (100% identity), while this conservation is lost in regions upstream of -500 bp of the *HGF* promoter (56).

Since Stat3 activation by Src induces specific gene expression and is required for cell transformation (88), we examined whether expression of Stat3 in the presence or absence of the activated c-Src mutant (SRC-Y527F) has any effect on *HGF* promoter activity. A reporter plasmid containing the -2.7 kb full length *HGF* promoter was co-transfected with a constant amount of the SRC-Y527F, and varying amounts of Stat3, expression plasmids. Expression of activated c-Src (SRC-Y527F) alone increased *HGF* transcription by about 2-fold (Fig. 5A). Likewise, expression of Stat3 alone increased *HGF* transcription by about 2-fold, and maintained a plateau value with even 0.05 μg of plasmid DNA. However, in cells co-expressing both the activated c-Src mutant and increasing amounts of Stat3, *HGF* transcription increased up to 5-fold. This result indicates that there is a co-operative effect between c-Src kinase activity and Stat3 protein in the regulation of *HGF* transcription.

The nonmalignant mammary epithelial cell line, HC11, shows at least a15-fold lower level of *HGF* transcription and no detectable HGF protein compared to SP1 carcinoma cells (data not shown). We therefore determined whether co-expression of c-Src and Stat3 can activate *HGF* transcription in HC11 cells. Expression of activated c-Src induced expression by about 4 fold (Fig. 5B). In contrast to SP1 cells, expression of Stat3 alone in HC11 cells did not significantly induce *HGF* transcription. However, when activated c-Src and Stat3 were co-expressed, *HGF* transcription was synergistically induced. These results suggest that increased c-Src kinase activity and Stat3 expression can over-ride the repression of *HGF* transcription in nonmalignant mammary epithelial cells.

To determine whether the c-Src responsive region of the *HGF* promoter is involved in the observed co-operative effect between c-Src and Stat3, the transcriptional activity of a mutant *HGF*

reporter lacking the c-Src responsive region (Δ1 HGF-luc) was compared to that of the full length (2.7 HGF-luc) *HGF* reporter. Each reporter construct was transfected into SP1 cells alone, or in combination with Stat3, and the activated c-Src (SRC-Y527F) mutant, expression plasmids. Expression of the activated c-Src mutant induced activation of the full length *HGF* promoter, but not of the deletion mutant (Δ1 HGF-luc) (Fig. 6). Similarly, Stat3 expression increased the activity of the full length *HGF* promoter, and only marginally affected that of the deletion mutant (Δ1 HGF-luc), this result suggests that Stat3 activates the *HGF* promoter. The level of induction due to Stat3 expression is even higher than that due to activated c-Src alone. This effect is probably due to a limiting amount of endogenous Stat3 in SP1 cells. When both Stat3 and activated c-Src were co-expressed, *HGF* promoter activity in the full length construct was strongly induced, this effect was not seen in the deletion mutant (Δ1 HGF-luc). These results show a cooperative effect between Stat3 and activated c-Src in the induction of *HGF* transcription, and imply the presence of specific Stat3 binding sites on the *HGF* promoter.

c-Src kinase regulates Stat3 activity through DNA binding and tyrosine phosphorylation:

Previous reports have found that c-Src activates Stat3 by inducing tyrosine phosphorylation of Stat3 and increasing its DNA binding affinity (88,96). We therefore examined the effect of c-Src kinase activity on Stat3 tyrosine phosphorylation in SP1 cells. We found that expression of activated c-Src induced Stat3 specific tyrosine phosphorylation while expression of the dominant negative c-Src has the opposite effect (Fig. 7). c-Src kinase activity similarly affected the DNA binding affinity of the Stat3 consensus sites on the HGF promoter (Fig. 8). We used electrophoretic mobility shift assays (EMSA) to examine the Stat3 consensus DNA binding affinity of nuclear protein

extracts from cells expressing different mutants of c-Src. Radiolabelled oligonucleotide probes with DNA sequences corresponding to the two Stat3 consensus binding sites in the region between -254 to –70 of the *HGF* promoter were used to detect putative Stat3 binding (Fig. 8). DNA binding activity of probes corresponding to both sites (-110 and -149) was detected in nuclear protein extracts of SP1 cells (lane 1 in Fig. 8A and 8B, respectively), and these binding activities were specific since the presence of the corresponding unlabelled probes abolished the binding (lane 2), while a probe with an unrelated DNA sequence had no effect (lane 3). In addition, when comparing lanes 1, 4 and 7(Fig. 9), it is apparent that there was less specific DNA binding in nuclear extracts from SP1 cells expressing SRC-RF than in control cells expressing no exogenous c-Src. Moreover, nuclear extracts from SP1 cells expressing activated c-Src had higher binding activity than that from untransfected cells. This finding indicates that the expression of SRC-RF reduces Stat3 DNA binding affinity whereas SRC-Y527F has the opposite effect. Thus specific binding to the Stat3 consensus sites correlates with the phosphorylation state of Stat3 in these cells.

Although there is a strong indication of Stat3 being the transcription factor binding to the c-Src responsive region of the *HGF* promoter, other Stat proteins (such as Stat1, Stat5A and Stat5B) can also bind to a Stat3 consensus site, albeit at lower levels (14,50,57). Therefore, antibodies against specific Stat proteins were used in supershift experiments to determine the composition of the DNA binding complex (Fig. 9). Nuclear extracts from SP1 cells were pre-incubated with antibodies against Stat1, Stat3, Stat5A or Stat5B prior to the addition of the radio-labelled probe. Both -110 (Fig. 9A) and -149 (Fig. 9B) probes formed DNA-protein complexes when nuclear extracts were added. However, only anti-Stat3 antibody could efficiently bind to these complexes to form a supershift band. Antibodies to Stat1 (data not shown), Stat5A or Stat5B did not retard the

DNA-protein complex further, despite the fact that these transcription factors were present in SP1 cells (data not shown). This observation suggests that Stat3 is preferentially involved in the DNA-protein complexes which bind to the c-Src responsive element.

Discussion

During normal breast development, HGF is expressed primarily by the mesenchymal cells while its receptor Met is expressed by the epithelial cells (2,97). However, HGF is expressed in regions of human invasive breast carcinoma, and in various breast carcinoma cell lines (19-23,27,31). During tumorigenesis HGF stimulates angiogenesis, invasion, and metastasis (8,10,59). Our laboratory (72) and others (26) have shown that HGF can stimulate survival of carcinoma cells. Therefore, acquired HGF expression leading to an HGF autocrine loop in breast carcinoma cells may be an important step during mammary tumorigenesis. However, the regulation of HGF expression in breast carcinoma cells is not very well understood, although some studies have been done in fibroblasts (41-43,54-56). In the present study, we examined the role of c-Src kinase, which shows increased activity in human breast cancer (74) in controlling HGF expression in breast carcinoma cells.

We previously described a mammary breast carcinoma cell line, SP1, which expresses both HGF and activated Met (25,76). In SP1 cells, several downstream signalling molecules, such as PI-3 kinase, phospholipase C γ and focal adhesion kinase, are constitutively phosphorylated on tyrosine residues in SP1 cells, consistent with the presence of an autocrine loop (67,68,76). We have also found that c-Src tyrosine kinase in SP1 cells is constitutively active and is required for several HGF-

dependent processes such as cell motility and anchorage-independent growth (74).

In this report, we examined the effect of c-Src kinase activity on HGF expression in SP1 cells. Inhibition of c-Src kinase activity in SP1, through either the presence of c-Src kinase inhibitors or the expression of a dominant negative mutant of c-Src, caused a decrease in *HGF* mRNA and protein levels. Expression of an activated c-Src kinase had the reverse effect. This finding suggests that c-Src is important in regulating the basal level of HGF transcription in epithelial and carcinoma cells, and can induce elevated expression of HGF. However, since inhibition of c-Src kinase activity cannot eliminate all of HGF basal expression, other transcription factors may play roles in HGF basal expression. Indeed, in our system, Sp1 transcription factor is essential in maintaining HGF basal level transcription but has no effect on c-Src induction of HGF expression (data not shown). Furthermore, aggregates of SP1 cells expressing the activated form of c-Src, in which HGF protein level was increased, showed spontaneous scattering when plated on plastic, compared to the parent cell line which required addition of exogenous HGF (Elliott, unpublished results). The higher level of endogenous HGF expression in SP1 cells expressing the activated form of c-Src may be sufficient to induce spontaneous scattering of these cells. Together, these findings suggest that c-Src kinase activity is important in regulating HGF expression.

By using deletion mutants of the *HGF* promoter, we mapped the c-Src responsive element to -254 to -70 bp. Since there is significant homology among the mouse, rat and human *HGF* promoter between -500 and +1 (56,81), the regulation of HGF expression by c-Src kinase through this element is probably conserved among these species. Previous studies in fibroblast cells have demonstrated several transcription factors which regulate HGF expression: C/EBP (-4 bp) (41), an epithelial cell-specific repressor (-16 bp) (53), Sp1/Sp3 (-318 bp) (43), estrogen receptor (ER) (-872 bp) (42) and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) (-860 bp) (42). Transgenic mouse studies showed that 0.7 kb of the *HGF* promoter exhibited the same expression pattern as the full length (2.7 kb) (5). Although in our system we observed that Sp1/Sp3 maintain the basal level expression of HGF in breast carcinoma cells, these sites are not responsible for c-Src induced expression of HGF (data not shown). The C/EBP site appeared to have no transcriptional activity *in vivo* (5). Binding sites for ER and COUP-TF are likely to be involved in estrogen-induced expression of HGF since the upstream sequence between -2.7 and -0.7 kb has been shown to be necessary for maximal inducibility of the *HGF* promoter (such as after partial hepatectomy) (5). However, the c-Src responsive region (-254 to -70 bp) described here has not been previously reported.

In the c-Src responsive region of the *HGF* promoter there are two consensus binding sites for Stat3 (at -110 and -149), both of which are completely conserved among human, mouse and rat. Our results showed that Stat3, in cooperation with c-Src kinase, can activate *HGF* promoter, this activation is completely dependent on the presence of these Stat3 binding sites and implies a role of Stat3 as a downstream effector of c-Src kinase. We therefore examined the mechanism by which c-Src regulates Stat3 activity in SP1 carcinoma cells. Stat3 has been shown to be regulated by both

tyrosine and serine phosphorylations (49,80,91). Although there is no direct evidence that Stat3 is phosphorylated directly by c-Src, some reports suggest that c-Src and Stat3 interact physically (11,13). Therefore, it is possible that c-Src regulates Stat3 through tyrosine phosphorylation. Our results showed that expression of a dominant negative form of c-Src reduced tyrosine phosphorylation of Stat3 and the expression of constitutively active c-Src mutant had the opposite effect. In addition, we found that the formation of a DNA-protein complex with the two Stat3 binding sites in the c-Src responsive elements was dependent on the level of c-Src kinase activity in the cells.

Stat2, -4 and -6 are not normally expressed in mammary tissues (4,36,45,47,48,66,82,83), and are therefore unlikely to be involved in the formation of DNA-protein complexes in SP1 cells. Both Stat1 and Stat3 have been shown to be activated by c-Src in fibroblast cells when they are stimulated with various growth factors (11,14,69), while Stat5 is expressed and activated during mammary development (37,46). Moreover, both Stat3 and Stat5 have been found to be constitutively active in cells transformed by v-Src, v-Abl and other oncoproteins (7,13,17,60,96). Therefore, other Stat proteins cannot be ignored as part of the complex. Supershift studies with antibodies against specific Stat proteins allowed us to identify Stat3, and exclude Stat1, -5A or -5B, as a component of the DNA-protein complex. Furthermore, since there is only one DNA complex formed with each probe and each probe can effectively abolish DNA-protein complex formation with the other (data not shown), the same DNA binding protein(s) must be involved in binding to each of these regions. Since Stat3 protein binds as dimers to its binding sites, it is reasonable to assume that Stat3 dimers are binding to both sites in the c-Src responsive region. Together, these observations suggest that c-Src kinase may regulate Stat3-dependent transcriptional activation

activity through direct or indirect tyrosine phosphorylation of Stat3 resulting in increased DNA-binding ability.

In contrast to SP1 carcinoma cells, the non-malignant mammary epithelial cell line, HC11, showed a very low level of *HGF* transcription with no detectable HGF protein. Furthermore, expression of activated c-Src (Y527F) had very little effect on *HGF* transcription in HC11 cells, possibly due to the presence of the epithelial cell-type specific repressor (53). However, co-expression of Stat3 and activated c-Src caused a strong synergistic induction of *HGF* transcription in HC11 cells, implying that the lack of c-Src kinase activity and the low level of activated Stat3 may be limiting for *HGF* transcription in HC11 cells. Increased activities of these proteins can possibly over-ride the repression by the cell-type specific repressor and allow expression of HGF in epithelial cells. Interestingly, we found that fibroblast cells, which normally express HGF, also require c-Src kinase activity to regulate HGF expression, and that this regulation of HGF is dependent on the same region of the *HGF* promoter as our breast carcinoma cell model (data not shown). These results suggest a similar regulation pattern between fibroblast cells that express HGF endogenously, and carcinoma cells, which acquire the ability to express HGF. It is possible that during epithelial-mesenchymal transition, epithelial cells acquire different genetic mutations leading to the activation of c-Src kinase. For example, increased expression and activity of HER2/Neu, an EGF-like receptor tyrosine kinase, in breast carcinoma cells has been shown to activate c-Src kinase (18,69). Activation of c-Src, in turn, may lead to a de-repression of HGF expression, giving these cells growth an advantage compared to non-transformed epithelial cells. This step may be an important initial step in tumorigenesis.

Here we have reported the regulation of *HGF* gene expression by c-Src kinase activity via

Stat3 activation in breast carcinoma cells. Although many reports have indicated that increased Src kinase activity (particularly through the expression of v-Src) can activate gene expression via Stat3, in this study we identify a target of elevated activity of c-Src kinase in breast carcinoma cells. There is recent evidence suggesting that an HGF autocrine loop can provide selective survival and growth advantage to carcinoma cells and that over-expression of HGF can be a reliable indicator of poor survival of breast cancer patients (94). Our findings therefore provide an important link between breast cancer progression and HGF expression, and suggest that the c-Src/Stat3 pathway regulating HGF expression can be a potential target for therapy in breast cancer treatment.

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Figure Legends

Figure 1. Treatment with the c-Src family kinase inhibitor PP2 decreases *HGF* mRNA level and transcription.

Panel A: Prestarved SP1 cells were incubated with the Src family kinase inhibitor PP2 at the concentrations indicated. After 24 hours, cells were lysed and total RNA was extracted. The amount of *HGF* mRNA in each sample was quantitated using RT-PCR with HGF-specific primers and primers for GUS B (see Materials and Methods). The amount of *HGF* mRNA was normalized to GUS B mRNA, and the level of *HGF* mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments +/- range.

Panel B: SP1 cells were transfected with a reporter plasmid containing the 2.7 kb fragment of the *HGF* promoter driving expression of the luciferase gene (2.7 HGF-luc). A β-galactosidase expression plasmid was co-transfected in each group for normalization to account for differences in transfection efficiency. After 24 h of incubation, PP2 was added at the concentrations indicated, and the cells were incubated for an additional 24 h, lysed and assayed for luciferase activity. Luciferase activity of each sample was expressed as percentage of untreated (control) cells. Values represent the mean +/- SD of triplicate samples. The experiment was done twice with similar results.

Figure 2. Ectopic expression of c-Src kinase mutants in SP1 cells.

SP1 cells were transfected with expression vectors containing activated c-Src (SRC-Y527F) or dominant negative c-Src (SRC-RF) or an empty expression vector (SP1). After 48 hours, cells were lysed. Equal amounts of the lysates were immunoprecipitated with anti-c-Src antibody. Half of the

immunoprecipitates was used to detect c-Src kinase activity using enolase as a substrate (top panel). The other half was subjected to western blotting with anti-Src antibody to determine total c-Src protein content in the immunoprecipitates (middle panel), and then reprobed with monoclonal anti-chicken c-Src (EC10) antibody to detect the level of ectopic expression of each c-Src mutant (bottom panel).

Figure 3. c-Src kinase activity modulates *HGF* mRNA and protein levels in SP1 cells.

Panel A: SP1 cells transfected with dominant negative Src (SRC-RF) or activated Src (SRC-Y527F) or empty vector (control) were prestarved overnight. PP2 (40 μM) was added to one plate of SP1 cells and incubated for an additional 24 h. A nonmalignant breast epithelial cell line HC11 was used as a negative control. Total RNA was isolated, and the amount of *HGF* mRNA in each sample was quantitated using RT-PCR and normalized to GUS B mRNA as described in Fig. 1. The level of *HGF* mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments +/- range.

Panel B: Serum-free conditioned media were collected for 24 h from HC11 cells, PP2-treated SP1 cells, and SP1 cells transfected as in Panel A. HGF protein from the conditioned media was purified using copper (II) affinity chromatography (71). The fraction containing HGF protein was concentrated in Microcon concentrators and subjected to denaturing SDS-PAGE. Recombinant HGF (100 ng) was included in one lane as a control. After electrophoresis, the proteins were transferred onto nitrocellulose and the blot was probed with anti-HGF antibody. Immunoreactive bands were revealed using Enhanced Chemiluminescence kit.

Figure 4. c-Src kinase responsiveness of *HGF* transcription requires the -254 to -70 bp region of the *HGF* promoter.

Panel A: SP1 cells were co-transfected with the *HGF*-luciferase reporter (2.7 HGF-luc), reporter constructs containing various deletions of the *HGF* promoter (see Panel B), and activated c-Src (SRC-Y527F), dominant negative c-Src (SRC-RF) or an empty expression vector (control). Luciferase activity was determined, and normalized in group as described in Fig. 1B. Values represent mean +/- SD of triplicate samples. The experiments were done three times using two different preparations of plasmid DNA with similar results.

Panel B: Schematic representation of the wildtype *HGF* reporter construct and the corresponding internal deletion mutants used in Panel A is shown. The name of each construct refers to the full length (2.7 kb) or truncated promoter sequences (1.2, 0.8, 0.5, 0.3, 0.1 kb) upstream of the transcriptional start site (indicated by arrow). In addition, constructs containing 0.5 kb sequence with an internal deletion of the region between –254 and –70 (0.5Δ), or the full length sequence containing internal deletion of regions between –254 and –70 (Δ1), -1231 and –755 (Δ2), or both (ΔΔ) were used.

Figure 5. Stat3 induces *HGF* transcription in co-operation with activated c-Src.

SP1 carcinoma cells (Panel A) and HC11 mammary epithelial cells (Panel B) were co-transfected with the 2.7 HGF-luc reporter and activated c-Src (SRC-Y527F) or an empty vector (control), in combination with varying amounts of Stat3. Luciferase activity was determined and expressed as a percentage of that in control cells as described in see Fig. 1B. Values represent the mean +/- SD of triplicate samples. The experiments were done twice with similar results.

Figure 6. Stat3 synergizes with activated c-Src to induce *HGF* transcription.

SP1 cells were co-transfected with the 2.7 HGF-luciferase reporter, or an internal deletion mutant (Δ1 HGF-luc), and a combination of activated Src (SRC-Y527F) and Stat3 as indicated in the figure. Transfections and luciferase assays were performed as described in Fig. 1B. Values represent the mean +/- SD of triplicate samples. The experiments were done four times with similar results.

Figure 7. c-Src kinase activity regulates phosphorylation of tyrosine residue 705 of Stat3

SP1 cells transfected with SRC-RF or SRC Y527F, or untransfected SP1 cells, were lysed. Equal amounts of proteins from each cell lysate were subjected to denaturing SDS-PAGE. The proteins were then transferred onto nitrocellulose and the blot was probed with antibody specific for phosphotyrosine 705 of Stat3 (Panel A). The blot was subsequently reprobed with anti-Stat3 (pan) antibody (Panel B).

Figure 8. c-Src kinase activity regulates binding of Stat3 to the Src-responsive region of the *HGF* promoter.

Nuclear extracts were prepared from SP1 cells transfected with SRC-RF, or SRC-Y527F, or untransfected cells. Equal amounts of each nuclear extract were used in binding studies with radiolabelled probes containing either the -110 (Panel A) or the -149 region (Panel B) of the *HGF* promoter. Ten-fold molar excess of an unlabelled probe containing the -110, -149 or a non-specific sequence (NS), respectively, was included in the binding reaction where indicated. The gel was fixed, dried, and analyzed using a Storm PhosphorImager as described in Materials and Methods. The arrow indicates the position of the protein-DNA complex.

Figure 9. Stat3 forms part of the DNA-protein complex at both the -110 and -149 consensus sites.

Nuclear extracts were prepared from SP1 cells as described in Materials and Methods. For supershift assays, nuclear extracts were incubated with anti-Stat3, Stat5A or Stat5B antibody on ice for 30 minutes prior to EMSA analysis. After incubation with labelled -110 (Panel A), or -149 (Panel B), probes, the reaction was subjected to non-denaturing PAGE. The asterisk indicates the position of supershift band.

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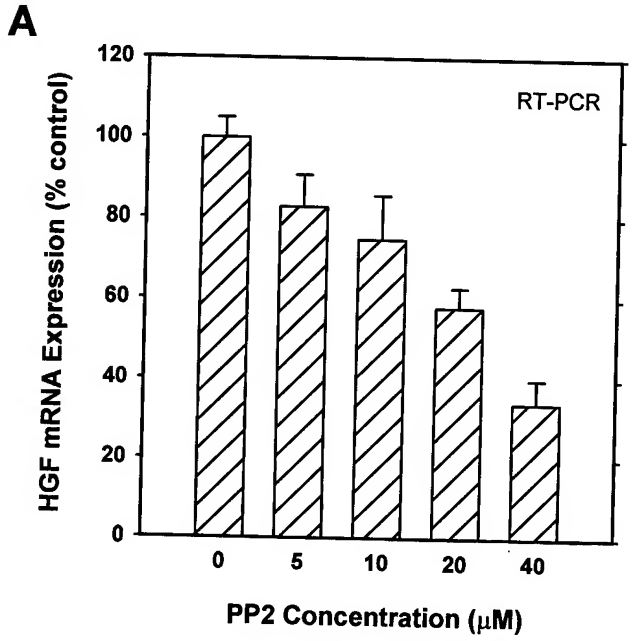
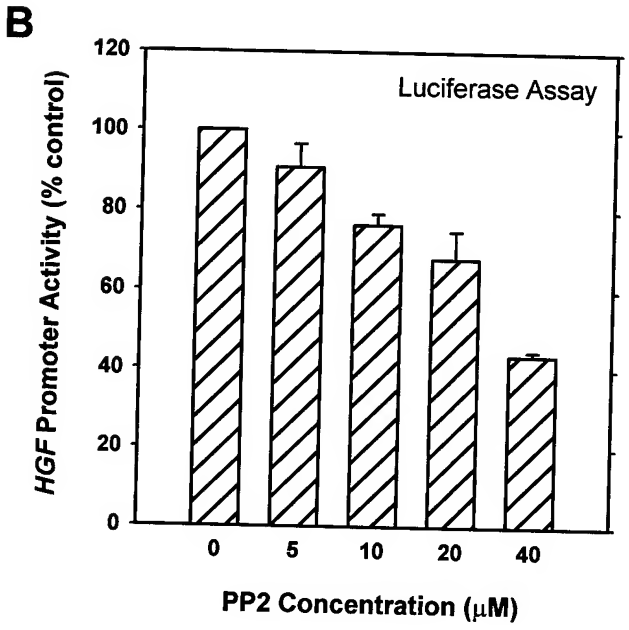
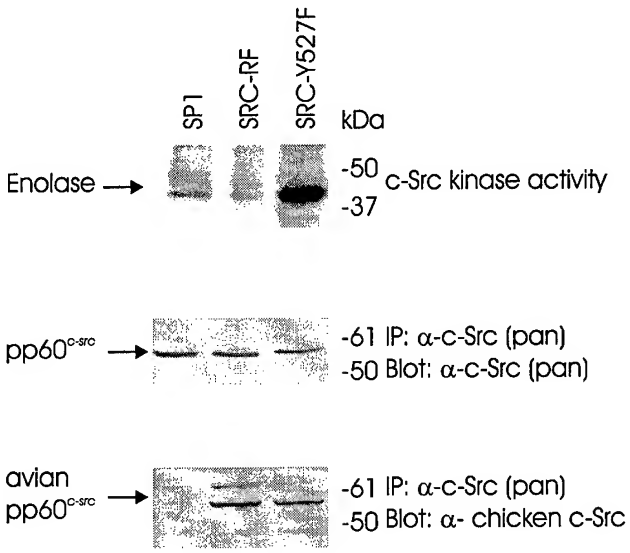
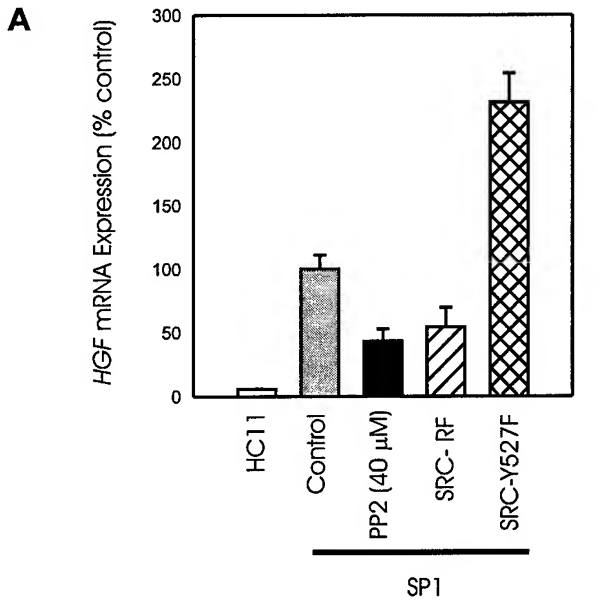
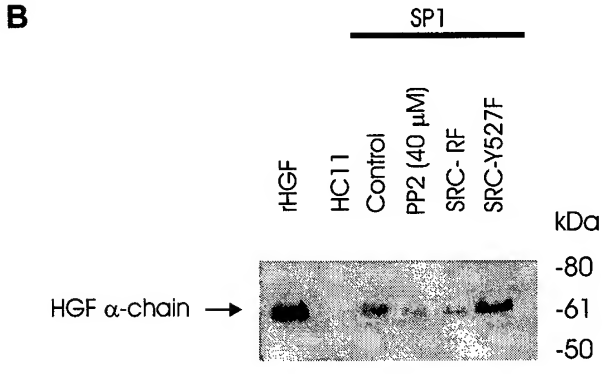

A
RT-PCR
HGF mRNA Expression (% control)
120
100
80
60
40
20
0
0
5
10
20
40
PP2 Concentration (μM)

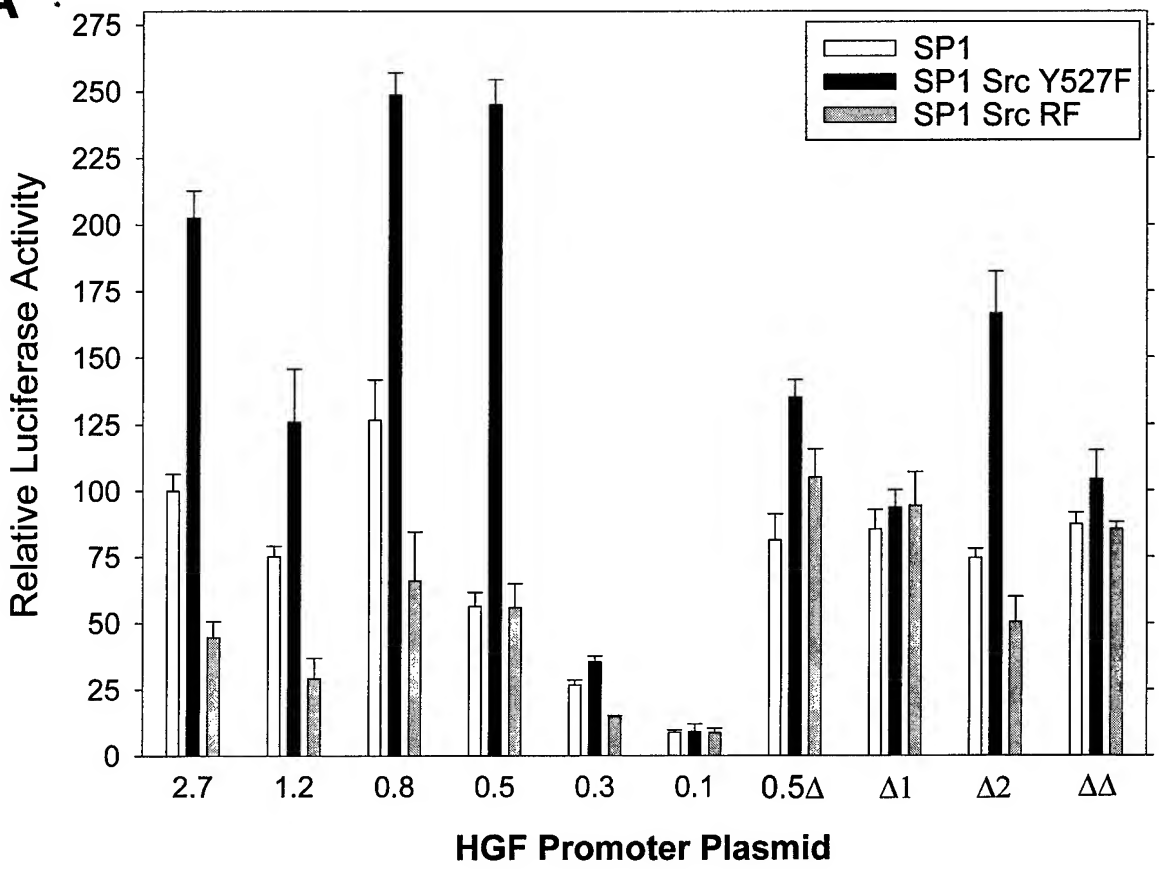

B
Luciferase Assay
HGF Promoter Activity (% control)
120
100
80
60
40
20
0
0
5
10
20
40
PP2 Concentration (μM)


SP1
SRC-RF
SRC-Y527F
kDa
Enolase
-50
-37
c-Src kinase activity
pp60c-src
-61 IP: α-c-Src (pan)
-50 Blot: α-c-Src (pan)
avian
pp60c-src
-61 IP: α-c-Src (pan)
-50 Blot: α- chicken c-Src

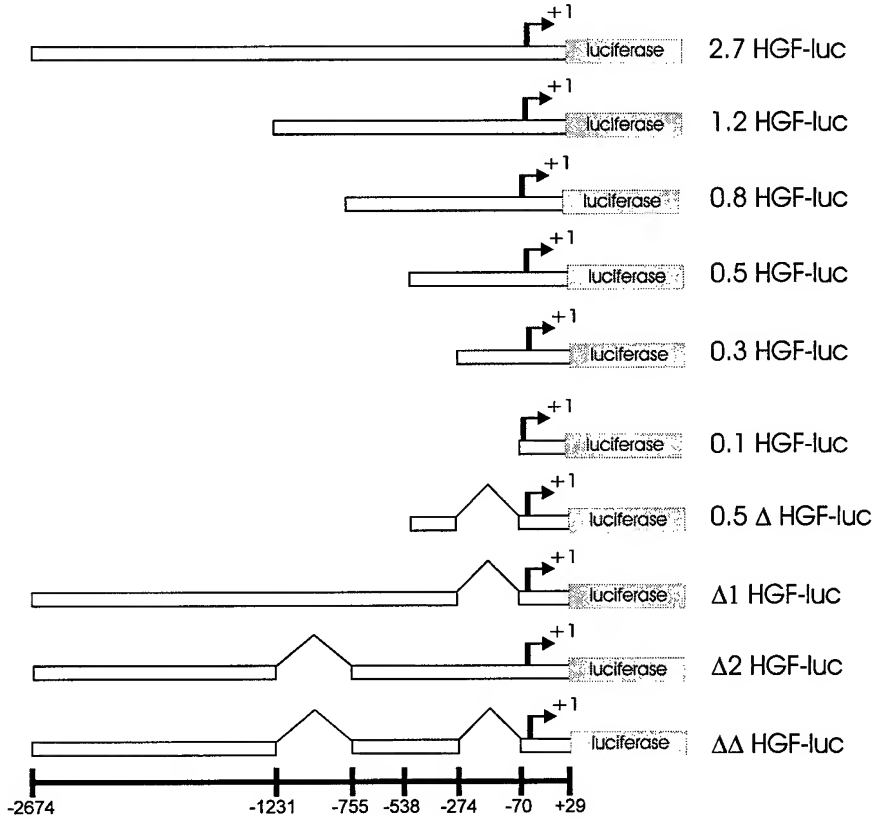

A
HGF mRNA Expression (% control)
300
250
200
150
100
50
0
HC11
Control
PP2 (40 μM)
SRC- RF
SRC-Y527F
SP1


B
SP1
rHGF
HC11
Control
PP2 (40 μM)
SRC- RF
SRC-Y527F
kDa
HGF α-chain
-80
-61
-50

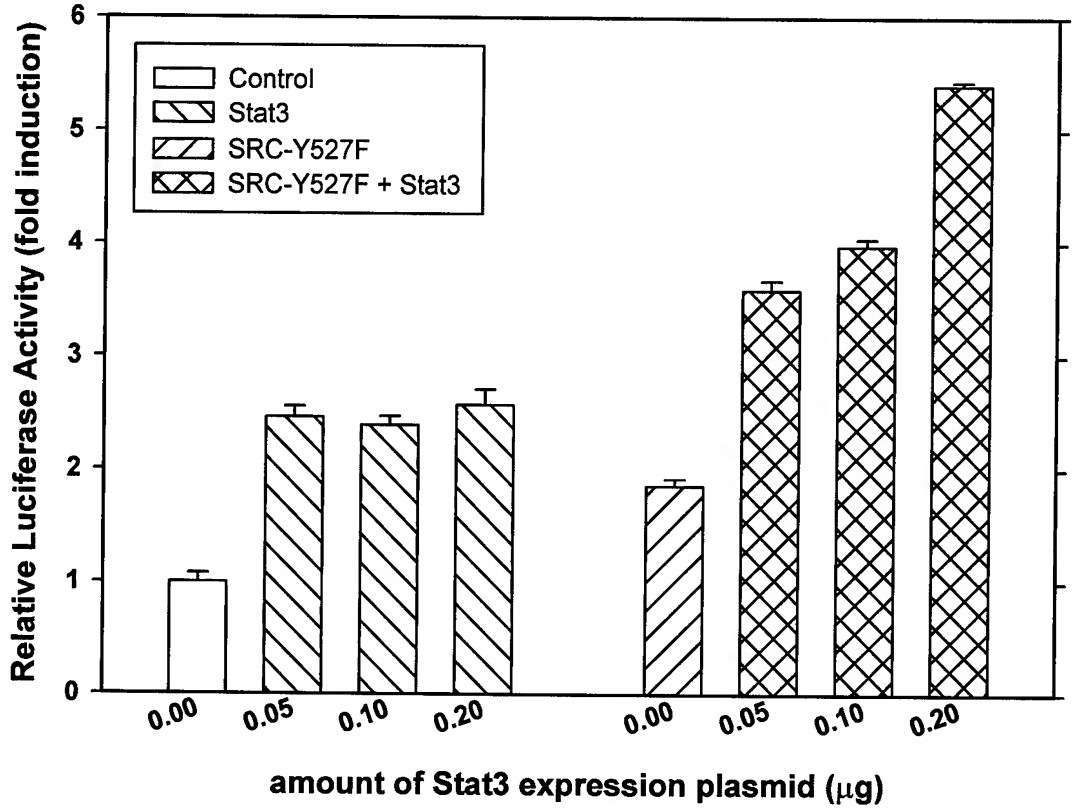
A


SP1
SP1 Src Y527F
SP1 Src RF
Relative Luciferase Activity
275
250
225
200
175
150
125
100
75
50
25
0
2.7
1.2
0.8
0.5
0.3
0.1
0.5Δ
Δ1
Δ2
ΔΔ
HGF Promoter Plasmid

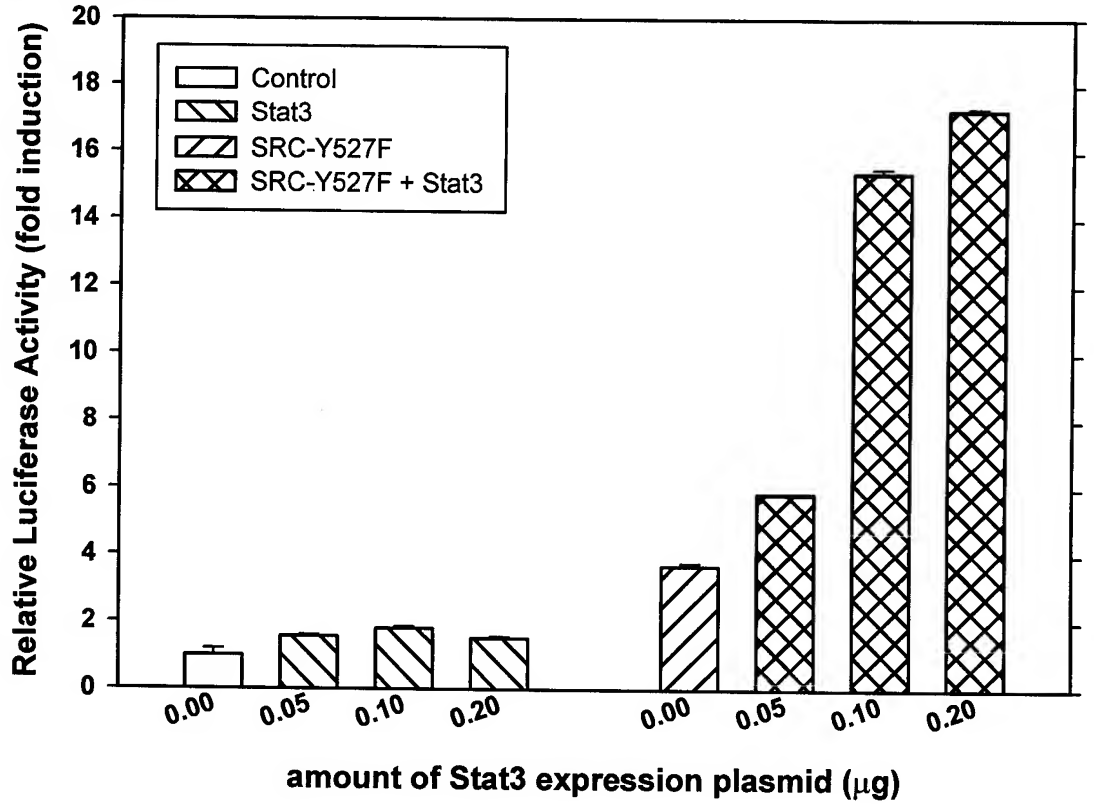
B

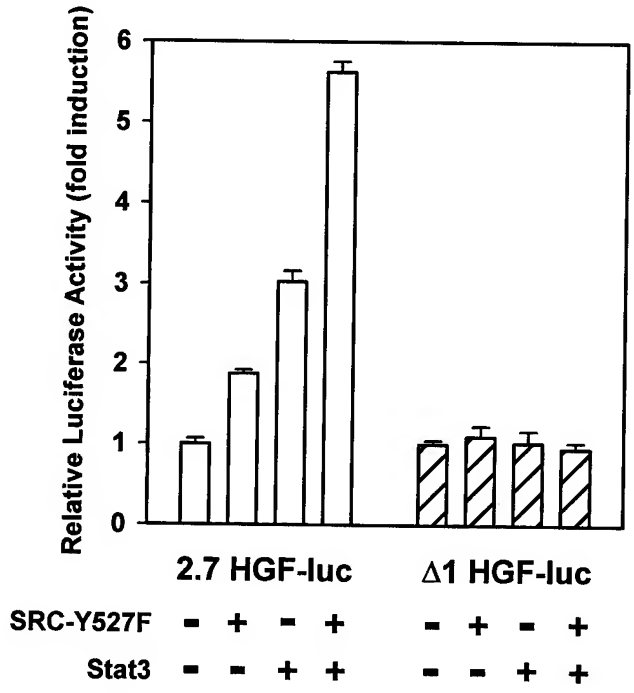

+1
luciferase
2.7 HGF-luc
1.2 HGF-luc
0.8 HGF-luc
0.5 HGF-luc
0.3 HGF-luc
0.1 HGF-luc
0.5 Δ HGF-luc
Δ1 HGF-luc
Δ2 HGF-luc
ΔΔ HGF-luc
-2674
-1231
-755
-538
-274
-70
+29

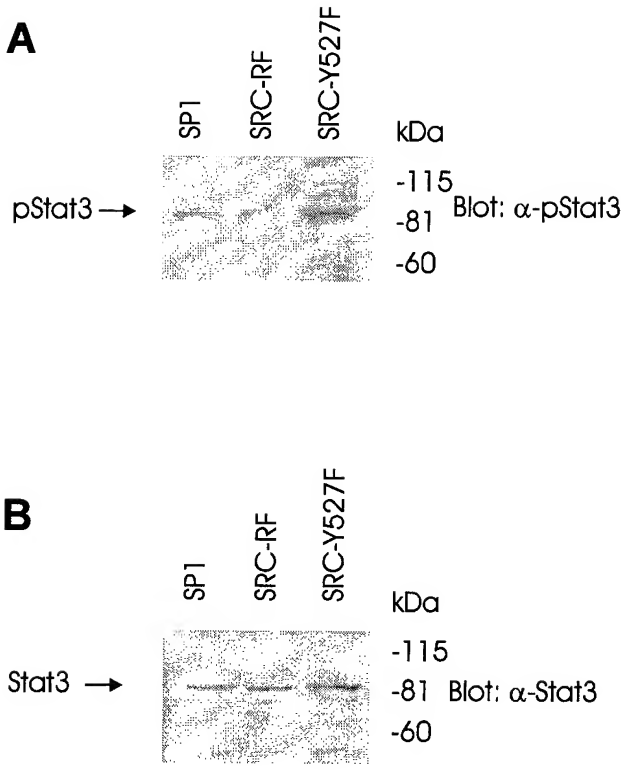
A. SP1 cells



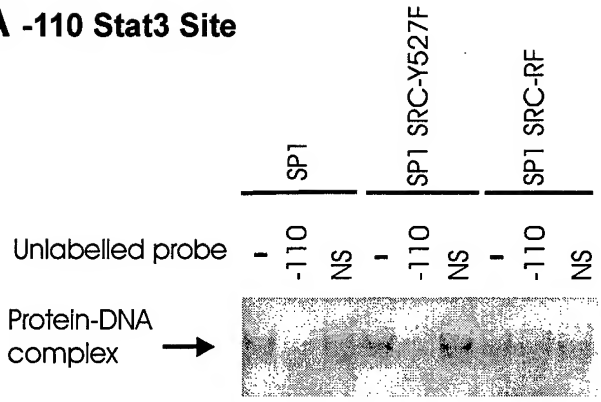
B. HC11 cells



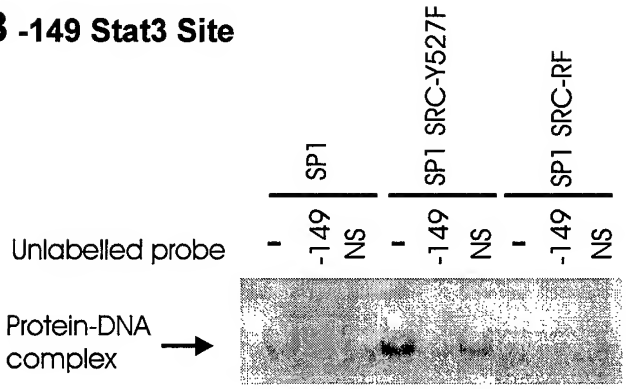

Relative Luciferase Activity (fold induction)
0
1
2
3
4
5
6
2.7 HGF-luc
Δ1 HGF-luc
SRC-Y527F - + - + - + - +
Stat3 - - + + - - + +

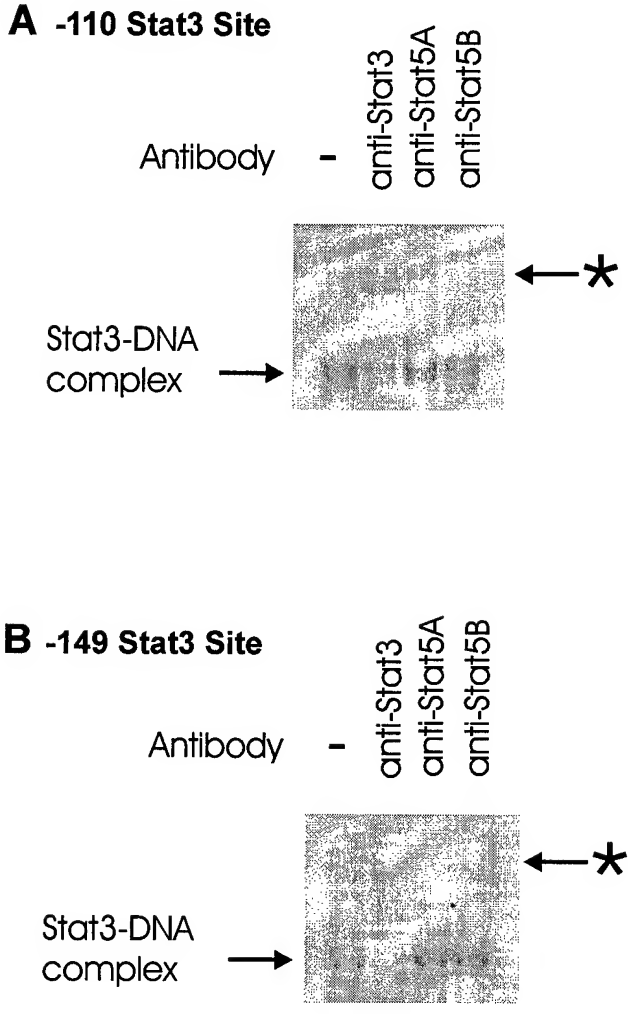

A
SP1
SRC-RF
SRC-Y527F
kDa
pStat3
-115
-81
-60
Blot: α-pStat3
B
SP1
SRC-RF
SRC-Y527F
kDa
Stat3
-115
-81
-60
Blot: α-Stat3

A -110 Stat3 Site



B -149 Stat3 Site




A -110 Stat3 Site
Antibody
-
anti-Stat3
anti-Stat5A
anti-Stat5B
Stat3-DNA complex
*
B -149 Stat3 Site
Antibody
-
anti-Stat3
anti-Stat5A
anti-Stat5B
Stat3-DNA complex
*

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c-Src Kinase Activity Is Required for Hepatocyte Growth Factor-induced Motility and Anchorage-independent Growth of Mammary Carcinoma Cells*

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Overexpression and amplification of hepatocyte growth factor (HGF) receptor (Met) have been detected in many types of human cancers, suggesting a critical role for Met in growth and development of malignant cells. However, the molecular mechanism by which Met contributes to tumorigenesis is not well known. The tyrosine kinase c-Src has been implicated as a modulator of cell proliferation, spreading, and migration; these functions are also regulated by Met. To explore whether c-Src kinase is involved in HGF-induced cell growth, a mouse mammary carcinoma cell line (SP1) that co-expresses HGF and Met and a nonmalignant epithelial cell line (Mv1Lu) that expresses Met but not HGF were used. In this study, we have shown that c-Src kinase activity is constitutively elevated in SP1 cells and is induced in response to HGF in Mv1Lu cells. In addition, c-Src kinase associates with Met following stimulation with HGF. The enhanced activity of c-Src kinase also correlates with its ability to associate with Met. Expression of a dominant negative double mutant of c-Src (SRC-RF), lacking both kinase activity (K295R) and a regulatory tyrosine residue (Y527F), in SP1 cells significantly reduced c-Src kinase activity and strongly blocked HGF-induced motility and colony growth in soft agar. In contrast, expression of the dominant negative c-Src mutant had no effect on HGF-induced cell proliferation on plastic. Taken together, our data strongly suggest that HGF-induced association of c-Src with Met and c-Src activation play a critical role in HGF-induced cell motility and anchorage-independent growth of mammary carcinomas and further support the notion that the presence of paracrine and autocrine HGF loops contributes significantly to the transformed phenotype of carcinoma cells.

Evidence supports a role of hepatocyte growth factor (HGF)[1] and its receptor, the product of the *met* protooncogene, in both normal (1, 2) and malignant (3–5) epithelial cell development. In addition, a majority of human breast cancers show increased expression of HGF and Met (6–8), and this high level of HGF expression correlates with recurrence and poor patient survival (9). Met is also overexpressed in several other human cancers, including ovarian (10), melanoma (11), colon carcinomas (12), and osteosarcomas (13). Collectively, these observations suggest that activation of Met by overexpression, gene amplification, or establishment of an HGF autocrine loop may contribute to growth and development of mammary carcinomas. Previous studies demonstrated that co-expression of HGF and Met (4, 14), as well as expression of a constitutively active Met (Tpr-Met) in NIH-3T3 fibroblasts (15, 16) directly leads to cell transformation and tumorigenicity. However, the molecular mechanism by which HGF binding to its receptor elicits cell transformation is not fully understood.

A number of cytoplasmic signaling proteins, such as phosphatidylinositol (PI) 3-kinase, Grb2, Shc, Ras, and c-Src, have been shown to be involved in Met-dependent signal transduction pathways (17, 18). It is important to establish which of these signaling proteins regulate Met-dependent steps in tumor progression, because different signaling proteins may regulate various HGF-induced cellular functions, including mitogenic, motogenic, and morphogenic signals in target cells (18–22). The HGF-mediated signaling pathway is further complicated by the observation that the majority of SH2-containing cytoplasmic effectors bind to a single multifunctional docking site on the cytoplasmic domain of Met, whereas a second site is required for Grb2 binding (17, 18). Recent findings using a mutational approach demonstrated that different HGF-induced effects are regulated by these separate Met binding sites for cytoplasmic transducers (23–25) and that complementation in *trans* between these two binding sites is required for the invasive-metastatic phenotype (25). However, to study the role of specific SH2-containing cytoplasmic effectors in HGF receptor function, approaches to target individual cytoplasmic effectors are required. Recently, we (26) and others (27) have demonstrated that PI 3-kinase activity is required for HGF-induced mitogenic (26) and motogenic functions (27). These findings strongly argue that PI 3-kinase may play an important role in HGF-mediated growth of mammary carcinomas.

The tyrosine kinase c-Src is activated in response to HGF (17, 18) and other growth factors such as platelet-derived growth factor (PDGF) (28–30), fibroblast growth factor (31),

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[1] The abbreviations used are: HGF, hepatocyte growth factor; PI, phosphatidylinositol; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; PDGF, platelet-derived growth factor.

and epidermal growth factor (32). c-Src kinase activity is known to modulate cell proliferation (33, 34), spreading (35, 36), and migration (36–38) in many cell types; these functions are also regulated by HGF (19–23). c-Src kinase activity is increased 4-fold in human breast cancer (39, 40) and is also elevated in Neu-induced mouse mammary carcinomas in transgenic mice (41, 42). Activation of c-Src tyrosine kinase in transgenic mice induces mammary epithelial hyperplasias and is required, but is not sufficient, for induction of mammary tumors in polyoma virus middle T-transgenic mice (42, 43). Altogether, these observations support the notion that increased c-Src kinase activity in mammary carcinomas plays an important role in mammary tumor growth and development. However, the role of c-Src kinase in HGF-induced functions in mammary carcinoma cells is not clearly known.

To analyze whether c-Src kinase is involved in HGF-induced mammary carcinoma cell growth, we used a mouse mammary carcinoma cell line, SP1, which expresses HGF and tyrosine-phosphorylated Met, thereby generating an autocrine HGF loop in these cells (44). Our current results demonstrate that c-Src kinase activity is elevated in SP1 cells, compared with nonmalignant Mv1Lu epithelial cells. The increased activity of c-Src kinase correlates with its ability to associate with tyrosine-phosphorylated Met. We therefore examined the effect of expressing a dominant negative mutant form of c-Src on c-Src kinase activity and HGF-induced cell motility and anchorage-independent growth of SP1 carcinoma cells. Taken together, our findings show that c-Src kinase activation plays a significant role in HGF-induced cell motility and anchorage-independent growth, characteristics of the transformed phenotype.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-sheep IgG conjugated to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (Westgrove, PN). Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KT). Rabbit anti-c-Src IgG, anti-Met (mouse) IgG, and anti-PLC-γ1 IgG were obtained from Santa Cruz Biotechnology (San Diego, CA).

Tissue Culture and Cell Lines—Mv1Lu cells are members of a mink lung epithelial cell line obtained from ATCC (Rockville, MA). Maintenance medium for Mv1Lu cells was Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% FBS. The SP1 tumor cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma and expresses HGF and Met. The characteristics of the SP1 cell line have been described elsewhere (45, 46). Maintenance medium for SP1 cells was RPMI 1640 (Life Technologies, Inc.) supplemented with 7% FBS (Life Technologies, Inc.).

Cell Transfection—cDNAs encoding wild type *c-src* (SRC) and a dominant negative double mutant of *c-src* (SRC-RF) with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F) ligated into the pRc/CMV plasmid (Invitrogen, San Diego, CA) carrying the neomycin resistance marker were obtained from Dr. J. Brugge (47). SP1 cells expressing the mutant c-Src and wild type c-Src were established using the stable transfection LipofectAMINE (Life Technologies, Inc.) method (48). Briefly, SP1 cells were grown to 80% confluence. The DNA (1 μg) was mixed with LipofectAMINE reagent (9 μl) in 200 μl of serum-free medium and was incubated for 15 min at room temperature. Before transfection, cells were washed once with 2 ml of serum-free medium. For each transfection, the mixed DNA and LipofectAMINE were combined with 0.8 ml of serum-free RPMI 1640 medium, and the cells were incubated with this transfection mixture. After 5 h of incubation, an equal volume of RPMI/14% FBS was added to the transfection medium, and incubation proceeded for an additional 24 h. For most experiments, pooled transfected cells selected with G418 (450 μg/ml) were used. In one experiment, SP1 cells were transfected with SRC-RF or SRC, and clones were isolated and tested for Src kinase activity and colony forming efficiency.

Cell Proliferation and Colony Growth Assay—Cell proliferation was carried out as described elsewhere (45). Briefly, SP1 carcinoma cells and Mv1Lu cells were plated at 10^4 cells/well in 24-well plates under the various conditions indicated. DNA synthesis was measured by adding 0.2 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Oakville, ON, Canada) at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (ICN, Costa Mesa, CA), and [^{3}H]thymidine incorporation was measured in a scintillation counter (Beckman, Mississauga, ON, Canada). Results are expressed as the mean cpm/well ± S.D. of triplicates.

Colony growth assays were performed as described previously (49). Briefly, a solution of 1.2% Bactoagar (Difco Lab) was mixed (1:1) with 2× RPMI 1640, supplemented with FBS at final concentrations of 7 or 1% alone or with HGF as indicated, and layered onto 60 × 15-mm tissue culture plates. SP1 cells (10^3/2.5 ml) were mixed in a 0.36% Bactoagar solution prepared in a similar way and layered (2.5 ml/plate) on top of the 0.6% Bactoagar layer. Plates were incubated at 37 °C in 5% CO_2 for 8–10 days. Colonies were fixed with methanol, stained with Giemsa, and counted manually. Results are expressed as mean number of colonies per dish ± S.D. of quadruplicates.

Cell Motility Assay—To measure cell motility, Transwell culture inserts (8-μm pore size) (Costar, Toronto, ON, Canada) were coated uniformly with gelatin (0.25% w/v, Sigma, Oakville, ON, Canada) on both sides for 2 min at room temperature (50). Membranes were washed twice with serum-free RPMI 1640 medium and inserted into a 24-well culture plate (Costar, Toronto, ON, Canada) with 1 ml of RPMI 1640 containing 0.5 mg/ml bovine serum albumin (Life Technologies, Inc.). Cells were grown to 50% confluence, serum-starved overnight, and harvested in 5 mM EDTA. Cells (2×10^4/100 μl) were plated in the insert and incubated for 6–8 h at 37 °C. Following the incubation, excess medium was removed, and cells were fixed in 1% paraformaldehyde (Sigma) for 15 min and stained with hematoxylin (Fisher, Oakville, ON, Canada). Cells on the upper side of the membrane were removed by wiping with cotton. Cells on the under side of the membrane were counted using an inverted microscope with phase contrast illumination. Cell motility is expressed as the number of migrating cells per well. In a parallel study, a wounding assay was performed, as described previously (36). Briefly, monolayers of each cell type were "wounded" by scraping with an Eppendorf yellow tip, washed, and incubated alone or with HGF for varying times. Migration was assessed visually by the ability of cells to close the wounded area.

Immunoprecipitation and Western Blotting—Cells were grown to confluence and serum-starved for 24 h. Cells were rinsed with cold phosphate-buffered saline three times and lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4 °C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce). Equal protein amounts from each cell lysate were incubated with the indicated antibodies at 4 °C for 2 h or overnight. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with 3% skimmed milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and probed for 1 h with the indicated antibodies. The membrane was washed three times for 5 min each with TBST buffer, incubated with horseradish peroxidase-labeled secondary anti-rabbit or anti-mouse antibodies for 15 min, and washed three times with TBST for 10 min each time. Immune complexes were detected using ECL (Amersham).

In Vitro c-Src Kinase Assay—In most experiments, an *in vitro* c-Src kinase assay using enolase as a substrate was performed as described previously (51). Briefly, lysates from SP1 and Mv1Lu cells were prepared, and equal protein amounts from each cell lysate were immunoprecipitated with anti-c-Src IgG (Santa Cruz Biotechnology) as described above. The amount of anti-c-Src IgG was pre-determined to be in excess over c-Src protein, indicating that the majority of c-Src protein in cell lysates is immunoprecipitated (data not shown). One-half of each immunoprecipitate was subjected to SDS-PAGE under nonreducing conditions and Western blot analysis to confirm the amount of c-Src protein present. The other half of each immunoprecipitate was assayed for c-Src kinase activity, by incubating with 10 μl of reaction buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4), 1 μl of freshly prepared acid-denatured enolase (Sigma) (5 μg of enolase + 1 μl of 50 mM HCl incubated at 30 °C for 10 min then neutralized with 1 μl of 1 M PIPES, pH 7.0), and 10 μCi of [γ-^{32}P]ATP. After 10 min of incubation at 30 °C, reactions were terminated by the addition of 2× SDS sample buffer, and samples were subjected to 8% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature and drying for 2 h at

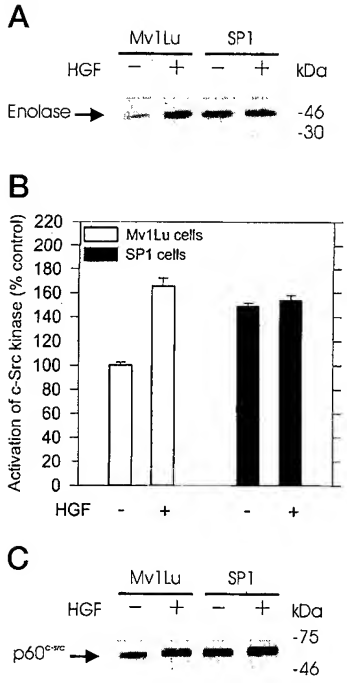

FIG. 1. **c-Src kinase activity is elevated in SP1 carcinoma cells compared with Mv1Lu epithelial cells.** Cell lysates were prepared from serum-starved Mv1Lu and SP1 cells treated without (−) or with (+) HGF (40 ng/ml) for 10 min and were immunoprecipitated with anti-c-Src IgG. Immunoprecipitates were subjected to an *in vitro* kinase assay using enolase as a substrate, and kinase activity was measured as described under "Experimental Procedures." *A*, autoradiogram showing ^{32}P-labeled enolase. *B*, quantitation of autoradiogram using PhosphorImager. Results are expressed as the percentage of cpm in untreated Mv1Lu cells (100%), normalized to the amount of c-Src protein in *C*. The means ± range of two experiments are shown. Similar results were obtained using the c-Src kinase family-specific cdc2 peptide as substrate (data not shown). *C*, Western blot analysis of immmunoprecipitates in *A*, probed with anti-c-Src IgG.

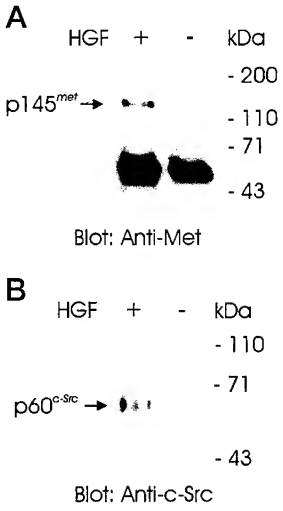

FIG. 2. **c-Src kinase binds to tyrosine-phosphorylated Met.** Cell lysates derived from serum-starved Mv1Lu cells treated without (−) or with (+) HGF (40 ng/ml) for 15 min were immunoprecipitated with anti-c-Src IgG (*A*) or anti-Met IgG (*B*). The immune complexes were separated by 8% SDS-PAGE and immunoblotted with anti-Met IgG (*A*) or anti-c-Src IgG (*B*). Protein molecular mass standards are shown on the *right*. This experiment was done twice with similar results.

80 °C under a vacuum. Autoradiograms were produced and quantitated using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In some experiments (see Fig. 3), c-Src kinase activity was assayed according to Cheng *et al.* (52) using the c-Src tyrosine kinase family-specific cdc2 peptide substrate. Anti-c-Src or anti-Met immunoprecipitates prepared as above were incubated with 40 μl of a reaction buffer (100 mM Tris-HCl, pH 7.0, 0.4 mM EGTA, 0.4 mM Na_3VO_4, 40 mM $Mg(OAc)_2$), 5 μl of cdc2 peptide (Life Technologies, Inc., 250 μM/assay), 5 μl of cold ATP (25 μM), and 2.5 μCi of [γ-^{32}P]ATP. A control consisting of immunoprecipitation with anti-Met IgG under more stringent conditions with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) where c-Src would not be co-precipitated was also carried out. After 15 min of incubation at 37 °C, reactions were terminated by the addition of 20 μl of 40% trichloroacetic acid and incubated for an additional 5 min. Aliquots subsequently were blotted on to p81 paper (Whatman, Fisher, Ottawa, ON, Canada). The p81 paper was washed three times (5 min/wash) with 0.75% phosphoric acid and once with acetone at room temperature, and the radiolabeled c-Src kinase substrate was counted in a liquid scintillation counter.

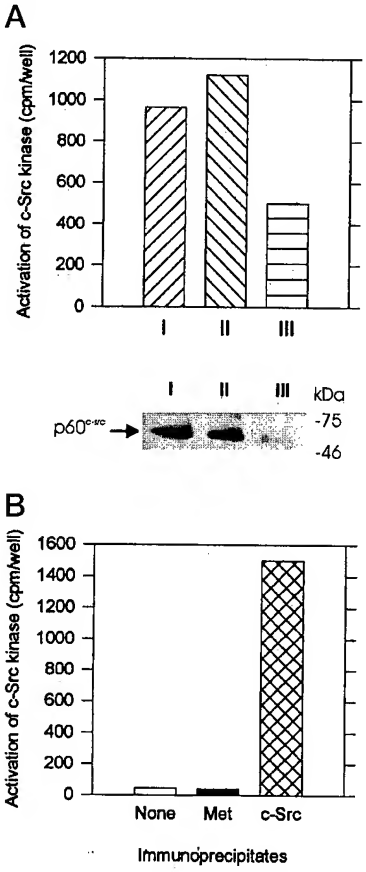

FIG. 3. **Detection of c-Src kinase activity in Met immunoprecipitates.** *A*, equal amounts of cell lysates derived from serum-starved SP1 cells were immunoprecipitated with anti-c-Src antibody (*bar I*) or anti-Met antibody (*bar II*). The supernatant from immunoprecipitates of anti-Met antibody was subsequently immunoprecipitated with anti-c-Src antibody (*bar III*). *In vitro* c-Src kinase activity was determined as described under "Experimental Procedures" using the c-Src kinase family-specific cdc2 peptide substrate. The amount of radiolabeled cdc2 substrate was determined and plotted as c-Src kinase activity (cpm/well) (*top panel*). Half of each immunoprecipitate in the *top panel* was subjected to SDS-PAGE, and p60$^{c\text{-}src}$ protein in each sample was identified by immunoblotting with anti-c-Src antibody (*bottom panel*). *B*, equal amounts of cell lysates derived from serum-starved SP1 cells were immunoprecipitated with anti-Met antibody or anti-c-Src antibody under more stringent conditions with RIPA buffer to prevent co-precipitation of other proteins (see "Experimental Procedures"). The immunoprecipitates were used in an *in vitro* c-Src kinase assay with the c-Src kinase family-specific cdc2 substrate. As a control, a reaction containing no protein (*None*) was carried out concurrently. Results are plotted as c-Src kinase activity (cpm/well) as in *A*. Anti-Met immunoprecipitates under these more stringent conditions showed no significant phosphorylation of the cdc2 substrate.

In Vitro Met Kinase Assay—Cell lysates from SP1 and Mv1Lu cells were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG as described above. Immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase

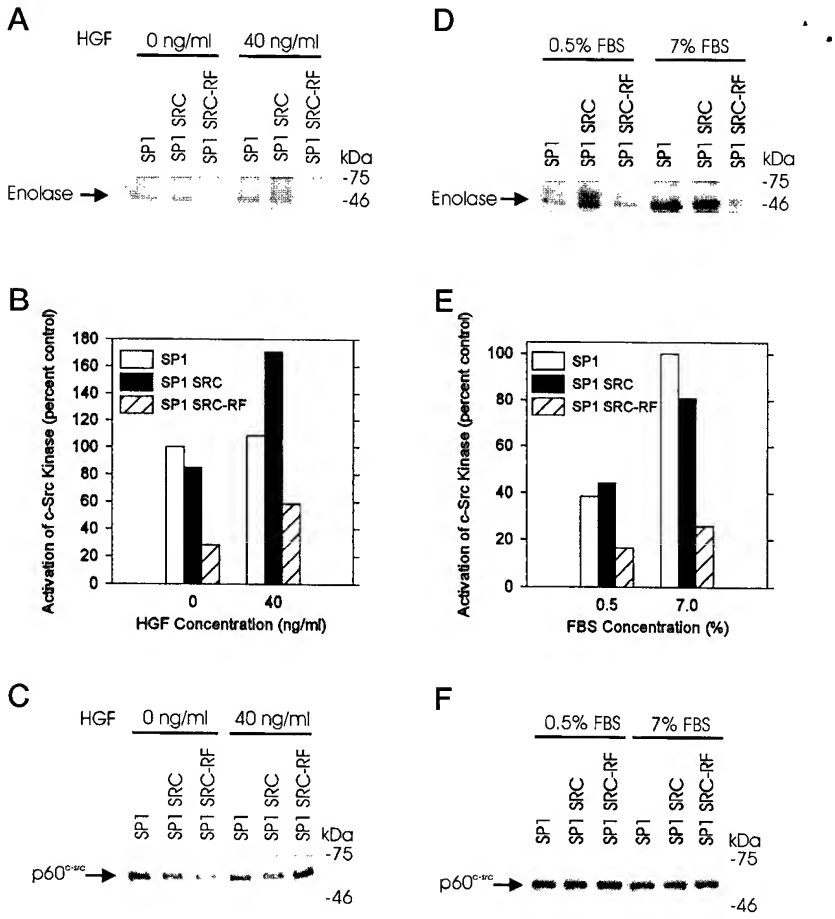

FIG. 4. **Effect of transfected dominant negative SRC-RF on Src kinase activity in SP1 cells.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were plated at 70% confluence and prestarved overnight. Cells in each group were then cultured alone, with HGF (40 ng/ml), or with 0.5 or 7% FBS, and an *in vitro* c-Src kinase assay using enolase as a substrate was performed as described under "Experimental Procedures." *A* and *D*, autoradiograms showing ^{32}P-labeled enolase. *B*, and *E*, quantitation of autoradiogram using densitometry. Results are normalized to amount of c-Src protein in *C* and *F*. *C*, and *F*, Western blot analysis of immmunoprecipitates in *A* and *D*, probed with anti-c-Src IgG. This result is representative of five experiments.

buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4). *In vitro* Met kinase activity was determined by incubating immunoprecipitates with 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP at 30 °C for 10 min. The reaction was stopped by addition of 2× SDS sample buffer containing 5% β-mercaptoethanol. Samples were boiled for 3 min and subjected to 7% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing and drying as described above. Autoradiograms were produced and quantitated using a Storm PhosphorImager (Molecular Dynamics).

RESULTS

Detection of Elevated c-Src Tyrosine Kinase Activity in SP1 Carcinoma Cells—SP1 carcinoma cells express HGF and tyrosine-phosphorylated Met, consistent with an HGF autocrine loop in these cells (44). To test the possibility that activation of c-Src kinase may be involved in Met-induced signaling pathways, we measured the kinase activity of c-Src in SP1 carcinoma cells and an HGF-sensitive epithelial cell line, Mv1Lu. c-Src kinase activity was measured by the capacity of c-Src immunoprecipitates from these cells to tyrosine phosphorylate the substrate, enolase. c-Src immunoprecipitates from serum-starved SP1 cells showed a pronounced elevated kinase activity, which increased only slightly following treatment with exogenous HGF (Fig. 1). In contrast, c-Src kinase activity in Mv1Lu cells was highly dependent on stimulation of cells with exogenous HGF (Fig. 1). The levels of c-Src kinase activity observed correlated with the constitutive tyrosine phosphorylation of Met (44) and *in vitro* Met kinase activity (data not shown) in SP1 cells, and the HGF-induced tyrosine phosphorylation of Met in Mv1Lu cells (Ref. 26 and data not shown).

Association of c-Src Kinase Protein and Activity with Activated Met—It is conceivable that the high level of c-Src kinase activity in SP1 cells, could have resulted from interaction of c-Src with activated Met due to an autocrine HGF loop in these cells (44). To test for interaction of c-Src kinase family proteins with activated *versus* nonactivated Met, we first examined the association of c-Src with Met in Mv1Lu cells that express Met but not HGF. Serum-starved Mv1Lu cells were incubated alone or with HGF, and cell lysates were immunoprecipitated with anti-Met IgG or anti-c-Src IgG. Protein precipitates were electrophoresed and subjected to Western blotting with anti-c-Src IgG or anti-Met IgG, respectively. As shown in Fig. 2 (*A* and *B*), an increased amount of c-Src protein was recovered from anti-Met immunoprecipitates and *vice versa* in cell lysates from HGF-treated Mv1Lu cells compared with untreated Mv1Lu cells. We also showed that association of c-Src kinase with Met occurred via the SH2 domain of c-Src and correlated with tyrosine phosphorylation of Met (data not shown). It should be noted that a trace amount of c-Src protein was detected in lysates of unstimulated cells immunoprecipitated with anti-Met IgG and blotted with anti-c-Src IgG, possibly due to incomplete starvation of these cells before HGF stimulation (Fig. 2*B*). Thus, stimulation with HGF causes increased association of c-Src protein with Met.

To determine whether elevated activity of c-Src kinase in SP1 cells correlates with its ability to associate with Met, serum-starved SP1 cells were immunoprecipitated with anti-Met IgG or anti-c-Src IgG, and immunoprecipitates were tested for the ability to tyrosine phosphorylate the c-Src kinase family-specific cdc2 peptide substrate (52). As shown in Fig. 3*A* (*bars I* and *II*), similar amounts of c-Src kinase protein and activity were recovered from immunoprecipitates of both anti-Met and anti-c-Src antibodies. In contrast, immunoprecipitates from anti-Met IgG under more stringent conditions with RIPA buffer where c-Src is not co-precipitated resulted in no signifi-

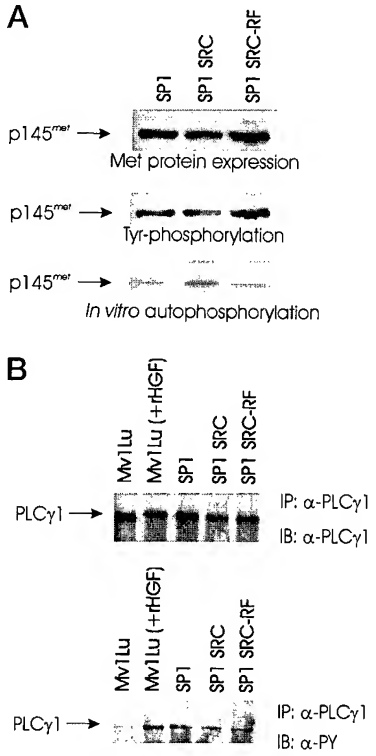

FIG. 5. **Expression of dominant negative mutant SRC-RF does not alter Met protein levels or activity and downstream signaling.** *A*, SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were prestarved overnight and lysed as described in the legend to Fig. 1. Equal amounts of protein from each lysate were concentrated on Microcon 10 filters (Amicon Inc., Beverly, MA) and analyzed by Western blotting with anti-Met IgG (*top panel*). The blot was stripped and reprobed with anti-phosphotyrosine antibody (*middle panel*). Cell lysates were also immunoprecipitated with anti-Met IgG, and immunoprecipitates were subjected to an *in vitro* Met kinase assay as described under "Experimental Procedures." The autoradiogram depicting ^{32}P-labeling of Met is shown (*bottom panel*). Relative band intensities and amount of ^{32}P labeling was determined using a Storm PhosphorImager. The relative amount of Met tyrosine phosphorylation (1.0, 1.0, or 1.0) or of *in vitro* Met autophosphorylation (1.0, 1.1, or 1.0) was not significantly different among the three cell lines. *B*, serum-starved SP1 cells transfected with SRC-RF or SRC and untreated SP1 cells were lysed as described in the legend to Fig. 1. Prestarved Mv1Lu cells untreated or treated with HGF (40 ng/ml) for 10 min were used as negative and positive controls, respectively. Equal amounts of protein from each lysate were immunoprecipitated with anti-PCL-γ1 IgG. Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-PCL-γ1 IgG (*top panel*) before being stripped and reprobed with anti-phosphotyrosine antibody (*bottom panel*). This experiment was done twice with similar results. *IP*, immunoprecipitation; *IB*, immunoblot.

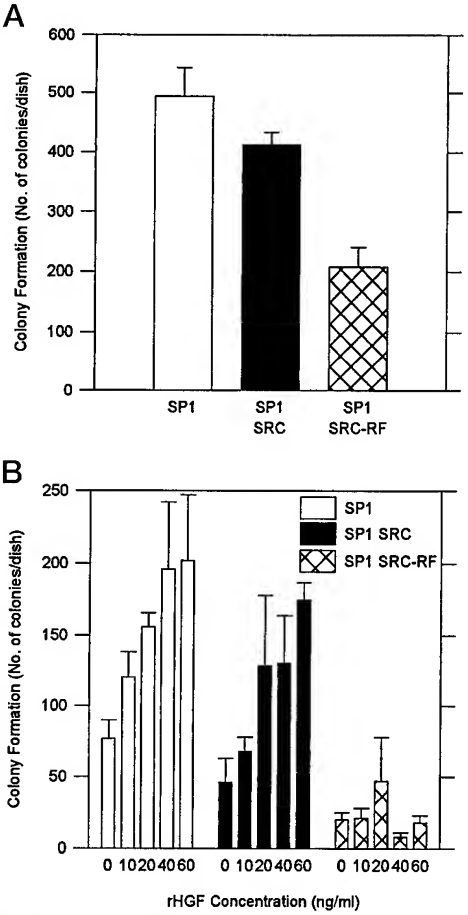

FIG. 6. **Effect of transfected dominant negative SRC-RF on growth of SP1 cells in agar.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were cultured (10^3 cells/dish) in 60-mm tissue culture plates in soft agar (0.36%) with RPMI 1640 medium supplemented with 7% FBS (*A*) or 1% FBS plus HGF at the concentrations indicated (*B*) as described previously (49). After 8 days, colonies were stained with Giemsa and counted visually. Results are expressed as the mean colony numbers ± S.D. of quadruplicate cultures. This experiment was done three times with similar results.

cant phosphorylation of cdc2 peptide (Fig. 3*B*), confirming the specificity of the cdc2 peptide as a substrate for c-Src (52). Thus a significant portion of c-Src kinase activity is associated with activated Met in SP1 cells. To further evaluate the contribution of c-Src association with Met, the supernatant from the immunoprecipitate of anti-Met IgG was immunoprecipitated for a second time with anti-c-Src IgG and subjected to the *in vitro* c-Src kinase assay. As shown in Fig. 3*A* (*bar III*), some c-Src kinase activity was detected in the Met-depleted SP1 cell lysate; however, it was with much lower activity, corresponding to the reduced amount of c-Src protein present. Immunoprecipitation of SP1 cell lysates with higher concentrations of anti-Met IgG and subsequently with anti-c-Src IgG showed a similar result (data not shown). These results demonstrate that the majority of c-Src kinase activity correlates with its ability to associate with Met in SP1 cells.

c-Src Kinase Activity Is Required for Colony Growth in Agar, but Not Cell Proliferation on Plastic—SP1 cells exhibit paracrine stimulation by HGF of colony growth in agar and proliferation on plastic (45, 49). To determine whether c-Src kinase activity is required for HGF-induced proliferation or colony growth in agar, an expression vector (SRC-RF) containing cDNA encoding a dominant negative double mutant of *c-src* with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F) (47) was stably transfected into SP1 cells. A control consisted of cells transfected with the same vector expressing wild type *c-src* (SRC). Uncloned (pooled) transfected cells were selected in G418-containing medium and assessed for c-Src kinase activity and HGF-induced functions. For *in vitro* c-Src kinase assays, immunoprecipitation with anti-c-Src IgG was carried out at antibody excess, indicating that the majority of wild type c-Src protein was present in immunoprecipitates. The results showed that c-Src kinase activity was strongly reduced in SRC-RF transfected SP1 cells compared with SRC transfected or untransfected cells incubated alone, or following stimulation with 40 ng/ml HGF or 7% FBS (Fig. 4). However, tyrosine phosphorylation of Met or an unrelated signaling molecule PLC-γ1 and *in vitro* autophosphorylation of Met remained unaffected in SRC-RF- or SRC-transfected SP1 cells, compared with untransfected cells (Fig. 5). These results demonstrate

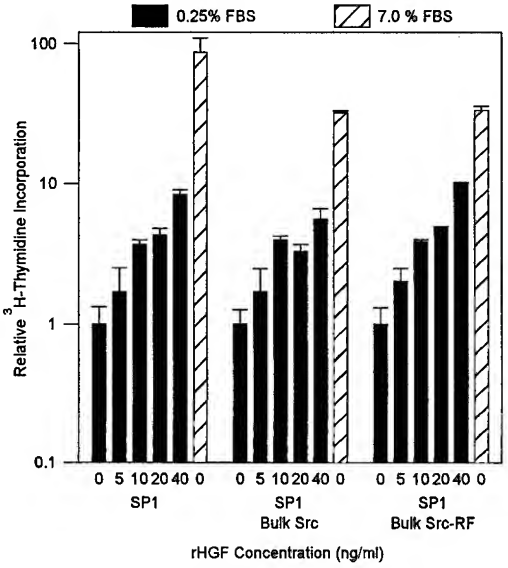

FIG. 7. **Effect of transfected dominant negative mutant SRC-RF on HGF-induced proliferation of SP1 cells on plastic.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were prestarved overnight, and each cell line was plated at 10^4 cells/well in 24-well plates in 0.25% FBS without or with HGF at the concentrations indicated. Controls consisted of cultures with 7% FBS. DNA synthesis was measured as described under "Experimental Procedures." Results are expressed as relative mean [^{3}H]thymidine incorporation compared with control (no HGF) (mean cpm/well ± S.D. of triplicates). This result is representative of four experiments.

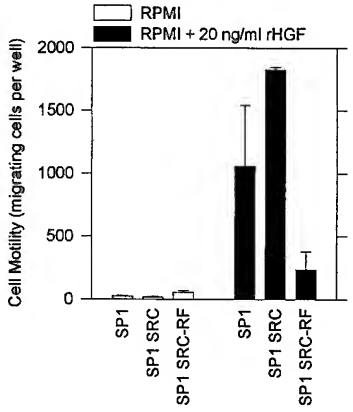

FIG. 8. **Effect of transfected dominant negative SRC-RF on HGF-induced cell motility.** SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were serum-starved overnight, and each cell line (2×10^4 cells) was plated into Transwell inserts (8-μm pore size) in 24-well plates in 0.5 mg/ml bovine serum albumin in RPMI without (*open bars*) or with (*closed bars*) HGF (20 ng/ml) as described under "Experimental Procedures." After 6–8 h of incubation at 37 °C, cells were fixed in 1% paraformaldehyde and stained with hematoxylin. Cells on the upper side of the membrane were removed by wiping with cotton. Cells on the underside were counted using an inverted microscope with phase contrast illumination. The results are expressed as the relative number of migrating cells/well (means ± range of two wells/point). This experiment was done twice with similar results. Similar results were obtained using a wound healing assay (data not shown).

specificity of the inhibitory effect of SRC-RF on c-Src kinase activity.

Expression of the dominant negative SRC-RF mutant in SP1 cells significantly inhibited FBS- and HGF-induced colony formation in soft agar, compared with SRC-transfected or untransfected SP1 cells (Fig. 6). Similarly, a subclone of SP1 cells expressing SRC-RF showed a marked reduction in colony formation, compared with a wild type SRC-transfected subclone or untransfected SP1 cells (data not shown). In contrast, SRC-RF-transfected SP1 cells showed no difference in HGF-induced or serum-induced proliferation on plastic, compared with SRC-transfected or untransfected SP1 cells (Fig. 7). Thus reduction of c-Src kinase activity in SRC-RF-transfected cells abrogated HGF- or serum-induced colony growth in soft agar but had no effect on cell proliferation on plastic.

c-Src Kinase Activity Is Required for HGF-induced Cell Motility—Because c-Src kinase activity has been shown to modulate cell motility in several cell types (36–38), we examined the role of c-Src kinase in HGF-induced cell motility in SP1 cells. Our results showed that HGF strongly stimulated motility of SP1 cells through collagen-coated porous membranes in a paracrine manner. HGF-induced motility was significantly reduced in SP1 cells transfected with dominant negative mutant SRC-RF, compared with SRC-transfected or untransfected cells (Figs. 8 and 9). Similar results were obtained using a wounding assay (data not shown). These results are consistent with a role of c-Src kinase in HGF-induced cell motility.

DISCUSSION

We (6) and others (7, 8) have previously shown that HGF and Met mRNA are strongly co-expressed in invasive carcinomas in human breast cancer. These findings suggest that signals transduced by activated Met confer survival and growth advantage to carcinoma cells during progression to metastasis. This concept is further supported by the observation that cells transfected with an activated version of *met* (*tpr-met*) acquire invasive and metastatic properties (15, 16). Unlike most other receptor tyrosine kinases, Met shows one high affinity binding site for the majority of SH2-containing cytoplasmic effectors, suggesting that these proteins bind Met in a competitive manner (23–25). Therefore, to study the role of specific SH2-containing cytoplasmic effectors in HGF receptor function, approaches to target individual cytoplasmic effectors are required.

To analyze downstream effector molecules in HGF-induced tumorigenic properties of mammary carcinoma cells, we have studied a mouse mammary carcinoma, SP1, which co-expresses HGF and Met (44). However, depending on culture conditions, both paracrine and autocrine effects of HGF have been observed in SP1 cells (44, 45, 49). In monolayer cultures, autocrine phosphorylation of Met at tyrosine in SP1 cells without addition of exogenous HGF was observed (44). In contrast, tyrosine phosphorylation of Met was reduced in suspended SP1 cells and can be restored by addition of exogenous HGF.[2] These observations suggest that the base level of Met activation may be influenced by extracellular environmental conditions, such as cell adhesion to various substrata (53), cell density effects on HGF expression and secretion (54), or proteolytic processing of pro-HGF to the biologically active form (55). In the present report, paracrine stimulation with exogenous HGF was required for optimal cell proliferation, motility, and colony growth in agar under serum-starved conditions. Previous studies showed that PI 3-kinase activity is elevated in SP1 cells and that its activity is required for HGF-induced proliferation in monolayer culture. Treatment of SP1 cells with wortmannin, a potent inhibitor of PI 3-kinase (56), or transfection of a dominant negative mutant of the p85 subunit of PI 3-kinase into these cells (26) inhibited HGF-induced cell proliferation in monolayer culture.

In the present report, we show that c-Src kinase activity is elevated in SP1 mammary carcinoma cells compared with nonmalignant Mv1Lu epithelial cells and is associated with Met. The elevated level of c-Src kinase activity in SP1 cells and its association with Met strongly suggest that this signaling mol-

[2] R. Saulnier and H. Qiao, unpublished observation.

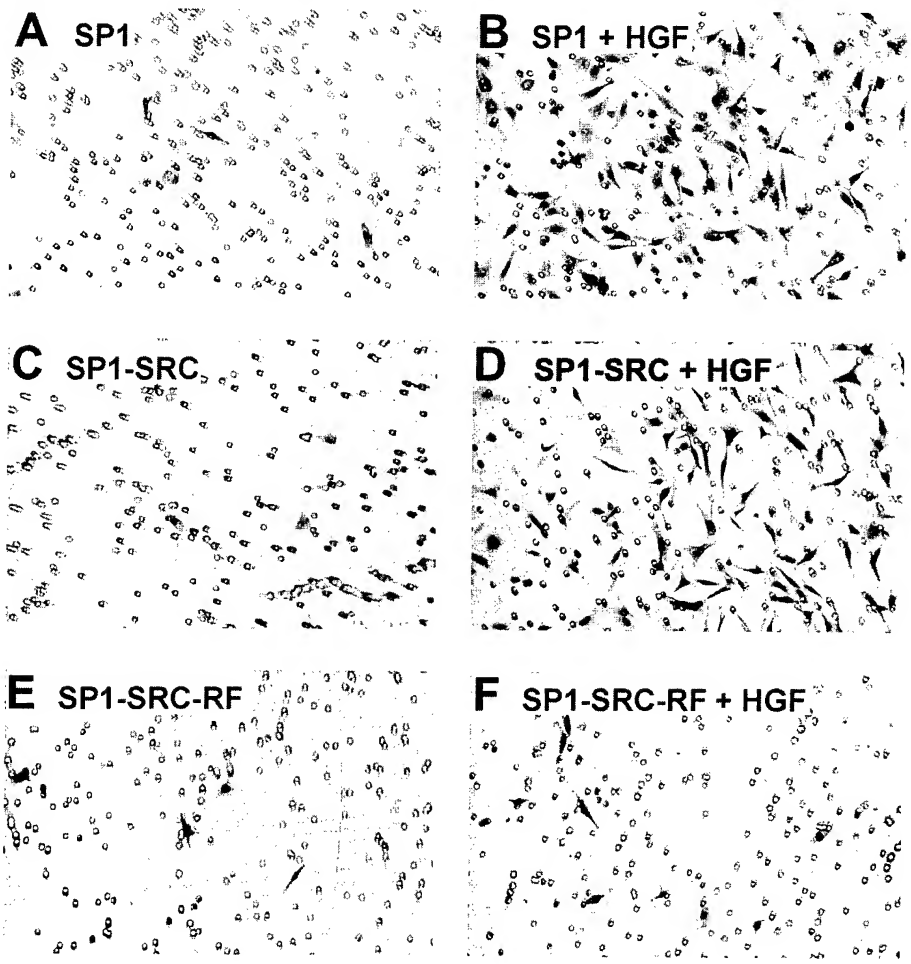

FIG. 9. **Photomicrographs of migrating SP1 cells transfected with SRC or SRC-RF or untransfected SP1 cells following HGF stimulation.** SP1 cells untransfected (*A* and *B*) and transfected with SRC (*C* and *D*) or SRC-RF (*E* and *F*) were serum-starved overnight and assessed for cell motility without (−) or with (+) HGF (20 ng/ml) as described in the legend to Fig. 8. After removing nonmigrating cells on the upper side of the membrane, membranes were mounted onto glass slides, and migrating cells were photographed using a Leitz microscope with phase contrast illumination. Photographs correspond to the groups shown in Fig. 8. Original magnification, 250×.

ecule may be involved in intracellular events triggered by HGF. This observation prompted us to test whether expression of a dominant negative mutant form of c-Src influences growth of SP1 cells. Expression of a dominant negative form of c-Src (SRC-RF) in SP1 cells showed no significant effect on HGF-induced cell proliferation on plastic but markedly inhibited HGF- or serum-induced colony growth in soft agar. Thus activation of c-Src kinase is essential for colony formation in agar by SP1 cells but appears not to be required for cell proliferation on plastic.

Other laboratories have reported variable effects of c-Src kinase on cell growth. In support of our observations, Demali and Kazlauskas (57) have shown that a mutant form of PDGF β-receptor that cannot bind or activate, c-Src, retains the ability to stimulate growth of fibroblasts on plastic or in agar in response to PDGF. In contrast, Courtneidge and co-workers (33, 58) have shown that microinjection of a kinase dead mutant c-Src or neutralizing antibodies that inhibit basal and stimulated c-Src kinase activity inhibited PDGF-dependent DNA synthesis in fibroblasts. Similarly, constitutive expression of c-Src mutants inhibited PDGF and epidermal growth factor-induced mitogenesis of mouse embryonal fibroblasts lacking c-Src (34). The apparent differences in the role of c-Src kinase in the above systems could be due to different levels of residual basal activity of c-Src kinase or the developmental and malignant status of the cells used. Our observation that anchorage-independent growth but not proliferation on plastic is inhibited in cells expressing dominant negative SRC-RF suggests that the reduced level of c-Src kinase activity in SRC-RF expressing SP1 cells is insufficient to support anchorage-independent growth, whereas proliferation on plastic remains unaffected. c-Src-independent signaling mechanisms may also promote HGF-induced proliferation of SP1 cells on plastic.

We have also shown that SP1 cells transfected with the dominant negative SRC-RF mutant showed reduced cell motility in response to HGF compared with SRC-transfected or untransfected SP1 cells. Thus c-Src kinase activity is required for HGF-induced cell motility in SP1 carcinoma cells, although complementary signaling molecules may also be involved. This observation reflects recent reports that c-Src kinase activity is required for epithelial cell scattering (38–40, 50) and organization of the cortical cytoskeleton (50). In addition, Richardson *et al.* (59) have shown that co-expression of c-Src in cells expressing the dominant negative C-terminal domain of focal adhesion kinase can reconstitute cell spreading and motility and induces tyrosine phosphorylation of paxillin. Together, these observations raise the possibility that HGF-induced c-Src kinase activity may regulate cell motility through the cytoskeletal complex. This possibility is currently being investigated.

There is now growing evidence that the c-Src family protein-tyrosine kinases are involved in signal transduction pathways that result in cell growth, adhesion, and differentiation. c-Src kinase activity is required for cell proliferation induced by platelet-derived growth factor, colony stimulating factor-1, and epidermal growth factor (51, 60), and increased c-Src kinase activity is associated with many cancers. These observations support the notion that increased c-Src kinase activity in mammary carcinomas may play an important role in mammary tumor growth and development. Our findings involving transfection of a dominant negative c-Src kinase-defective mutant into SP1 cells represent the first direct demonstration of a requirement for c-Src kinase activity in HGF-induced cell mo-

tility and anchorage-independent growth of carcinoma cells, although interactions with other signaling molecules may also be involved. These data strongly suggest that HGF-induced association of c-Src kinase with Met and its activation are important in growth and transformation of mammary carcinomas and further argue that paracrine and autocrine HGF loops play a significant role in the transformed phenotype of some mammary carcinomas.

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Cooperative Effect of Hepatocyte Growth Factor and Fibronectin in Anchorage-independent Survival of Mammary Carcinoma Cells: Requirement for Phosphatidylinositol 3-Kinase Activity[1]

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Abstract

Anchorage-independent survival and growth are critical characteristics of malignant cells. We showed previously that the addition of exogenous hepatocyte growth factor (HGF) and the presence of fibronectin fibrils stimulate anchorage-independent colony growth of a murine mammary carcinoma, SP1, which expresses both HGF and HGF receptor (Met; R. Saulnier *et al.*, Exp. Cell Res., *222*: 360–369, 1996). We now show that tyrosine phosphorylation of Met in carcinoma cells is augmented by cell adhesion and spreading on fibronectin substratum. In contrast, detached serum-starved cells exhibit reduced tyrosine phosphorylation of Met and undergo apoptotic cell death within 18–24 h. Under these conditions, the addition of HGF stimulates tyrosine phosphorylation of Met and restores survival of carcinoma cells. Soluble fibronectin also stimulates cell survival and shows a cooperative survival response with HGF but does not affect tyrosine phosphorylation of Met; these results indicate that fibronectin acts via a pathway independent of Met in detached cells. We demonstrated previously that inhibition of phosphatidylinositol (PI) 3-kinase activity blocks HGF-induced DNA synthesis of carcinoma cells (N. Rahimi *et al.*, J. Biol. Chem., *271*: 24850–24855, 1996). We now show in detached cells a cooperative effect of HGF and FN in the activation of PI 3-kinase and on the phosphorylation of PKB/Akt at serine 473. PI 3-kinase activity is also required for the HGF- and fibronectin-induced survival responses, as well as anchorage-independent colony growth. However, c-Src kinase or MEK1/2 activities are not required for the cell survival effect. Together, these results demonstrate that the PI 3-kinase/Akt pathway is a key effector of the HGF- and fibronectin-induced survival response of breast carcinoma cells under detached conditions and corroborate an interaction between integrin and HGF/Met signalling pathways in the development of invasive breast cancer.

Introduction

Basement membrane proteins and growth factors are important components of the tissue microenvironment that maintain survival and differentiation of normal epithelium (1). Disruption of basement membrane and aberrant expression of ECM[5] proteins occur during development of invasive carcinomas (2–4) and are associated with loss of epithelial polarity, increased cell survival, motility, and invasion (5). This process, known as epithelial-mesenchymal transition, is characteristic of the malignant phenotype and is considered an indicator of poor prognosis in many types of carcinomas. However, the mechanisms that promote survival of carcinoma cells during the invasive stage of tumor progression are not clearly known.

We (6) and others (7, 8) have shown that increased expression of HGF and its receptor, Met, occurs in invasive human breast cancer, particularly at the migrating tumor front (6), and that high levels of HGF and Met expression correlate with poor survival of breast cancer patients (9, 10). In addition, overexpression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (11, 12) or in transformed cell lines (13–15) promotes tumorigenic and metastatic properties. HGF is a multifunctional cytokine that stimulates mitogenic, motogenic, morphogenic and angiogenic functions in various cell types (reviewed in Ref. 16). Recent results also support a role of HGF as a survival factor during development of fetal liver (17), as well as in carcinoma cells treated with chemotherapeutic drugs (18, 19). Together, these findings imply that paracrine and autocrine activation of the HGF/Met signaling pathway may be an important

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[5] The abbreviations used are: ECM, extracellular matrix; HGF, hepatocyte growth factor; FN, fibronectin; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum.

regulatory step in survival and growth of invasive breast carcinomas.

In addition to altered growth factor responsiveness, remodeling of the ECM microenvironment through degradation and atypical expression of ECM proteins, such as FN, occur during progression to invasive carcinomas (5) and may affect cell survival and growth phenotypes (20–22). Indeed, adhesion to FN via $\alpha5\beta1$ and ligation of $\alpha v\beta3$ integrins have been shown to directly inhibit the death of Chinese hamster ovary cells (20) and human melanoma cells (23), respectively, under serum-starved conditions. Cell-ECM interactions have also been shown to collaborate with growth factor receptors (*e.g.,* epidermal growth factor receptor, Her2/Neu, PDGF β-receptor, and insulin-like growth factor receptor) in many biological processes such as growth and differentiation of various cell types (reviewed in Refs. 24–27). However, the majority of these studies were carried out in monolayer cultures, and their relevance to invasive and metastatic carcinoma cells is not clearly established.

In the present work, we examined the cooperative role of FN and HGF in regulating the survival of mammary epithelial and carcinoma cells under anchorage-independent conditions, characteristic of the invasive phenotype. We showed previously that exogenous HGF and the presence of FN fibrils promote anchorage-independent colony growth of a mammary carcinoma cell line, SP1, which expresses HGF and tyrosine-phosphorylated Met (28, 29). We now report that under serum-starved detached conditions, SP1 cells show reduced tyrosine phosphorylation of Met and undergo cell death, whereas the addition of HGF promotes tyrosine phosphorylation of Met and cell survival. Soluble FN also promotes cell survival and shows a cooperative survival effect with HGF. A similar cooperative survival response to HGF and FN was evident in a nonmalignant mammary epithelial cell line, HC11; however, a greater dependence on FN was observed. Previously, we (30) and others (31) had shown that PI 3-kinase, which regulates a number of cellular functions including morphogenesis (32), motogenesis (33, 34), and cell survival (35, 36), is required for HGF-induced DNA synthesis in monolayer cultures. Our results now show that the PI 3-kinase/Akt pathway (37) is a common downstream regulator of the cooperative survival effect of HGF and FN in carcinoma cells. In contrast, c-Src kinase, which is involved in cell spreading, migration, and anchorage-independent growth (38), and MEK1/2 have no effect on survival of carcinoma cells. Our findings provide new evidence for cooperativity of HGF and FN in survival of detached carcinoma cells via a signalling pathway independent of the focal adhesion complex. These results may prove useful in developing improved treatments of invasive breast cancer.

Results

Serum-starved Epithelial and Carcinoma Cells Undergo Apoptotic Cell Death under Anchorage-independent Conditions. Nonmalignant (HC11) and malignant (SP1) epithelial cells survive under anchorage-independent conditions in 7% FBS but die within 24–48 h after serum starvation (0% FBS; Ref. 29; data not shown). In contrast, both cell lines in monolayer culture survive in serum-starved conditions during the same time period. To examine the mechanism of cell death in cells maintained under serum-starved, anchorage-independent conditions, we used three different methods:

(*a*) Using acridine orange/ethidium bromide staining to assess nuclear morphology, we found that serum-starved, anchorage-independent cells develop irregularly shaped nuclei and condensation of chromatin within 24–48 h after serum starvation (Fig. 1, *A* and *B,* and data not shown). The majority of cells show uptake of ethidium bromide (red), indicating disruption of the plasma membrane, characteristic of late-stage apoptosis. In contrast, cells maintained in 7% FBS exclude ethidium bromide, indicating an intact plasma membrane, and show uniform green staining of nuclei with acridine orange. Similar results were found with two non-small cell lung carcinoma cell lines, A549 (which expresses Met but not HGF) and SK-Luci-6 (which expresses HGF but not Met; data not shown).

(*b*) Using an *in situ* end-labeling technique, we further demonstrated significant DNA fragmentation in serum-starved SP1 carcinoma cells, compared with cells in 7% FBS (Fig. 1, *C* and *D*).

(*c*) Using electron microscopy, we found that serum-starved SP1 carcinoma cells exhibit marked shrinkage and blebbing of the cell cytoplasm and dense condensation of the nuclear chromatin, in contrast with cells in 7% FBS (Fig. 1, *E* and *F*). Organelles and membranes in these cells also show good preservation.

Together, these characteristics indicate significant apoptotic cell death of serum-starved mammary epithelial and carcinoma cells in the absence of anchorage.

HGF and FN Stimulate a Cooperative Survival Response in Epithelial and Carcinoma Cells in the Absence of Anchorage. We have shown previously that HGF and FN promote anchorage-independent growth of SP1 cells (29). We therefore investigated whether soluble HGF and FN could provide survival signals to HC11 epithelial and SP1 carcinoma cells maintained in the absence of anchorage. Results from both cell staining and colorimetric assays show that soluble FN and HGF can promote survival of SP1 carcinoma cells in a dose-dependent manner (Fig. 2*A*). In contrast to FN, collagen type I and laminin do not promote survival of detached cells (data not shown). In addition, a cooperative increase in SP1 cell survival was observed in response to both HGF and FN at limiting concentrations, whereas a maximum cell survival with no demonstrable cooperative effect was observed in response to either HGF or FN at higher concentrations. A similar cooperative survival response to HGF and FN was observed in HC11 epithelial cells; however, these cells showed a greater dependence on FN for survival at all concentrations tested (Fig. 2*B*).

Soluble FN Acts Independently of Met Activation in the Survival Response of Detached Cells. The mechanism of soluble FN stimulation of cell survival may occur directly after integrin ligation and activation of downstream signaling mole-

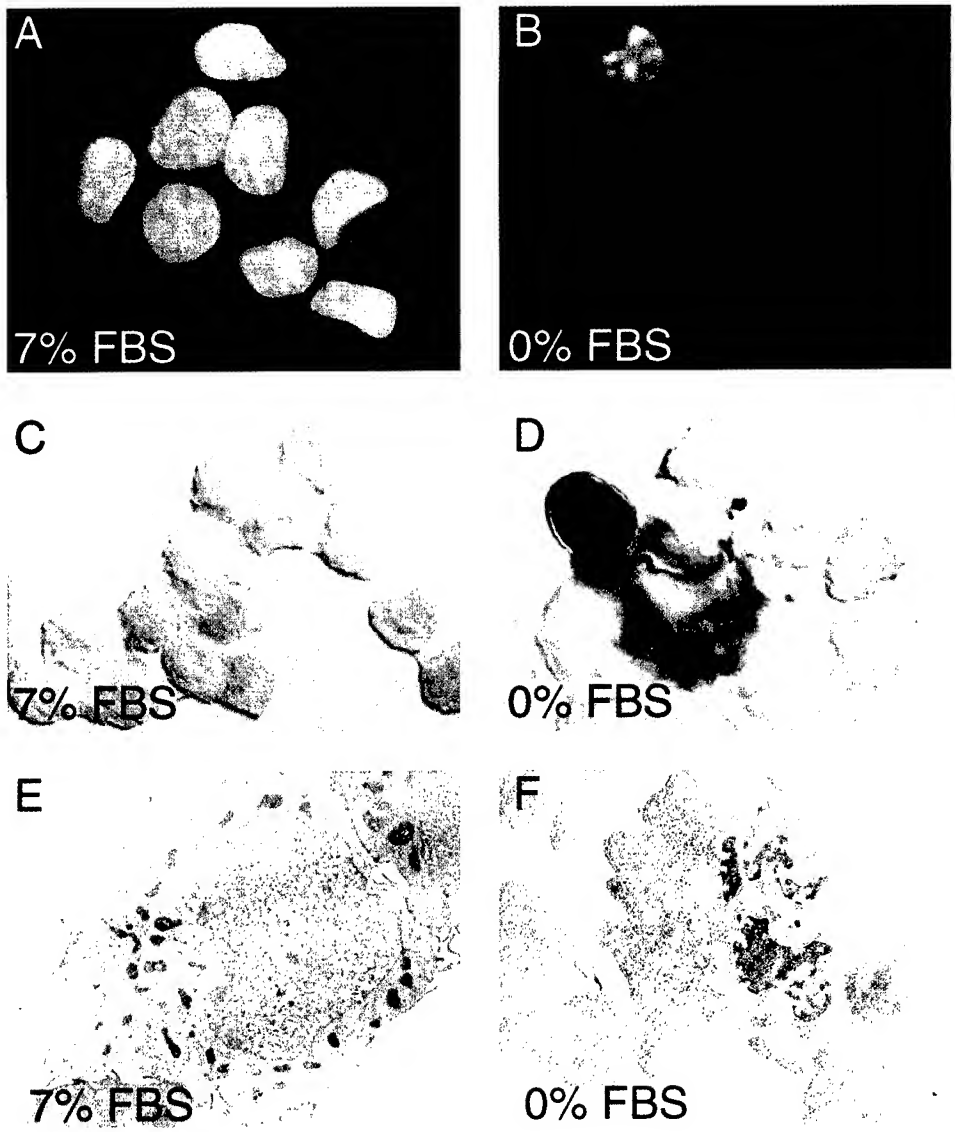

Fig. 1. Detached SP1 carcinoma cells show apoptotic cell death in the absence of serum. SP1 cells were prestarved overnight, subcultured in suspension on agar-coated plates in RPMI 1640 with 7% FBS (*A, C,* and *E*) or 0% FBS (*B, D,* and *F*) for 24 h, and assayed for apoptotic cell death in three ways as described in "Materials and Methods." *A* and *B,* cells were stained with acridine orange and ethidium. Fluorescence images were acquired separately for each stain from the same field with a Meridian confocal microscope and superimposed with MD4 Imaging Research software. Nuclei of viable cells stained uniformly *green*. Early apoptotic cells, in which plasma membranes are still intact, stained *green* but showed chromatin condensation as patches in nuclei. Late apoptotic cells, in which plasma membranes are disrupted, stained *red* with patches of condensed chromatin in nuclei. ×600. *C* and *D,* an *in situ* end-labeling technique was used to assay for DNA fragmentation. ×400. *E* and *F,* cells were fixed in gluteraldehyde and processed for electron microscopy. *E,* ×5000; *F,* ×8000.

cules (21) or indirectly by facilitating activation of Met (39, 40). We therefore examined the tyrosine phosphorylation level of Met in SP1 cells on FN substratum or in suspension with soluble FN. Our results showed that Met is constitutively phosphorylated at tyrosine residues in SP1 cells adhering to, and spreading on, FN substratum and shows a further increase after addition of HGF (Fig. 3*A*, and data not shown). However, the level of tyrosine phosphorylation of Met in SP1 cells is dramatically reduced in cells within 60 min after detachment and is restored within 15 min of incubation with HGF. The change in the level of tyrosine phosphorylation of Met in detached cells is not a result of protein loading because all lanes contained equal amounts of Met (Fig. 3*B*). In contrast, addition of soluble FN has no effect on the baseline level of Met tyrosine phosphorylation. Neutralizing anti-HGF IgG has no effect on FN-induced cell survival (data not shown). Together, these findings indicate that soluble FN acts independently of Met activation in the cell survival response. We have therefore examined the role of signaling molecules downstream of integrin and Met receptors in this system.

PI 3-Kinase Activity Is Required for HGF- and FN-induced Anchorage-independent Survival of SP1 Carcinoma Cells. We have shown previously that PI 3-kinase activity is required for HGF-induced proliferation of SP1 cells (30). PI 3-kinase is also involved in growth factor- and ECM-mediated survival of adherent cells in other systems (21, 35). We therefore examined the effect of HGF and FN on PI 3-kinase activity in SP1 cells in the absence of anchorage. Preliminary experiments showed that PI 3-kinase activity is reduced at least 2-fold in nonadherent, compared with adherent, SP1 cells (data not shown). Addition of HGF or FN alone at limiting concentrations marginally increases PI 3-kinase activity (Fig. 4, *A* and *B*), whereas addition of HGF and FN together at the same limiting concentrations results in a cooperative increase in PI 3-kinase activity. Higher concentrations of HGF or FN stimulate maximal PI 3-kinase activity (Fig. 4, *C* and *D*). In addition, a concomitant

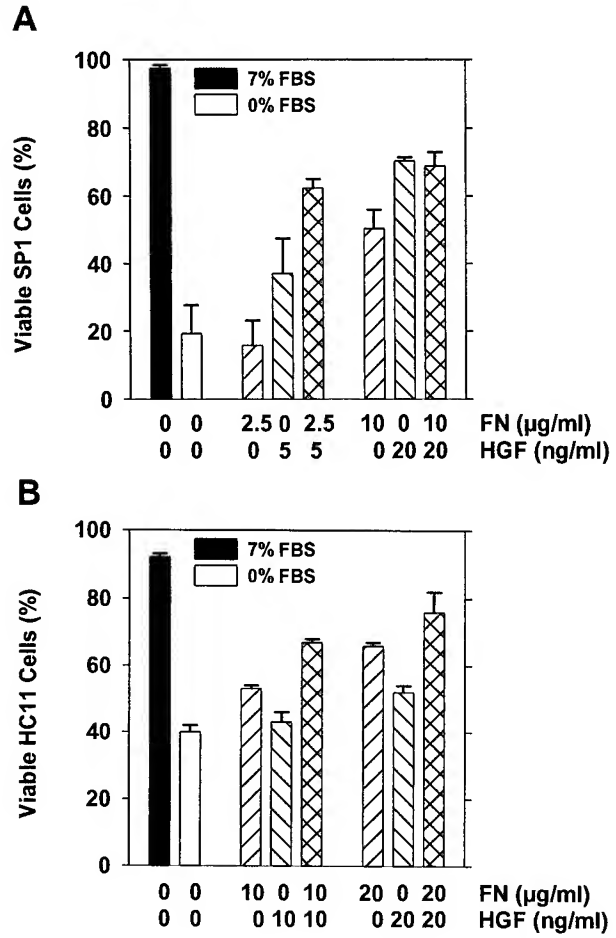

Fig. 2. Cooperative stimulation by HGF and FN of survival of SP1 carcinoma and HC11 epithelial cells under detached conditions. SP1 and HC11 cells were seeded in suspension culture without serum, and HGF, FN, or HGF + FN were added at the concentrations indicated. After incubation at 37°C for 24 h (SP1) or 48 h (HC11), cell survival was assayed with acridine orange/ethidium bromide staining. *A,* SP1 cell survival. *B,* HC11 cell survival. The results represent the mean ± range of duplicates and are representative of two experiments; *bars,* range of duplicates in one experiment.

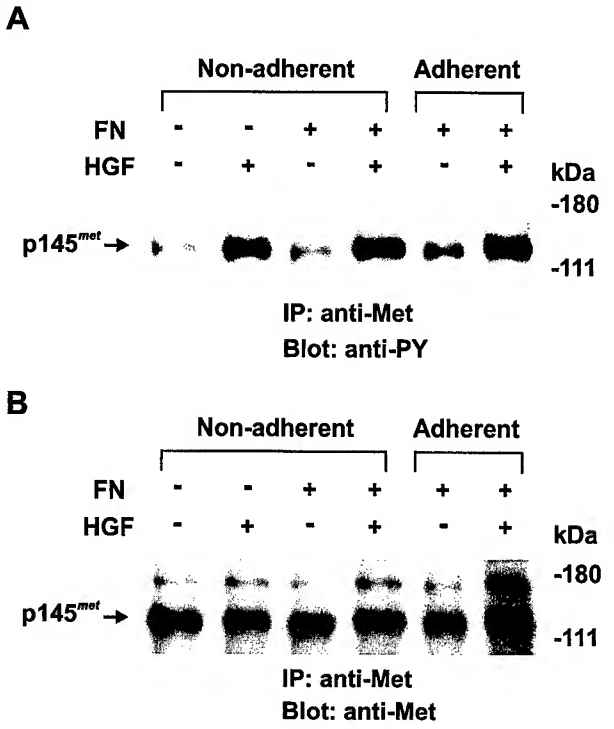

Fig. 3. Effect of cell adhesion on tyrosine phosphorylation of Met in SP1 cells. *A,* SP1 cells were prestarved overnight in serum-free medium and then either replated on FN-coated plates or kept in suspension for 45 min. HGF (20 ng/ml) or soluble FN (20 μg/ml) were added to the cells as indicated and incubated for an additional 15 min at 37°C. Cells were lysed, immunoprecipitated with anti-Met IgG, subjected to 6% reducing SDS-PAGE, and transferred to nitrocellulose, followed by Western blotting. The blot was probed with anti-phosphotyrosine antibody, and the bands were visualized with ECL reagents. *B,* the same blot was stripped and reprobed with anti-Met IgG to confirm that equal amounts of Met protein were loaded per lane.

cooperative increase in phosphorylation at serine 473 of PKB/Akt, which is an effector of cell survival downstream of PI 3-kinase (37), was also observed in response to HGF and FN at all of the concentrations tested (Fig. 5). Thus, HGF and FN stimulate PI 3-kinase activity and serine phosphorylation of PKB/Akt in a cooperative manner, corresponding to the cell survival response shown in Fig. 2.

To assess the role of PI 3-kinase in HGF- and FN-induced cell survival, we examined the effect of the PI 3-kinase inhibitor LY294002 (41), which was shown previously to block even very low basal levels of PI 3-kinase (42). Treatment with LY294002 inhibited HGF- and FN-induced survival (Fig. 6) in detached SP1 cells in a dose-dependent manner. A similar inhibition of serine phosphorylation of PKB/Akt was also observed (Fig. 5). As a control, we showed that LY294002 blocks PI 3-kinase activity in cells in monolayer culture with 7% FBS (Fig. 7*A*) and inhibits anchorage-independent colony growth in agar (Fig. 8) at the same concentrations that inhibit cell survival within 24 h. Treatment with LY294002 had no detectable effect on expression or tyrosine phosphorylation of Met in SP1 cells (Fig. 7*B*). Together, these results show that the PI 3-kinase pathway is an important regulator of the cooperative survival response of detached cells to HGF and FN.

c-Src Kinase and MEK1/2 Activities Are Not Required for HGF- or FN-induced Anchorage-independent Survival of SP1 Carcinoma Cells. We have reported previously that c-Src kinase activity is required for HGF-induced cell motility and anchorage-independent growth of SP1 cells (38). Furthermore, the Ras/MAPK pathway has been shown to regulate ECM-dependent cell survival (43). We therefore determined the role of c-Src kinase and MEK1/2 activities in survival of SP1 cells. SP1 cells transfected with a dominant-negative mutant of c-Src (SRC-RF) exhibited at least a 3-fold reduction in c-Src kinase activity compared with cells transfected with wild-type c-Src (SRC; Ref. 38). Under these conditions, the tyrosine phosphorylation levels of Met and phospholipase Cγ are unaffected (38). Our results now show no change in the survival response to HGF or FN of SP1 cells transfected with the dominant-negative SRC-RF mutant compared with wild-type SRC-transfected or untransfected cells (Table 1). Furthermore, we found that the MEK1/2 in-

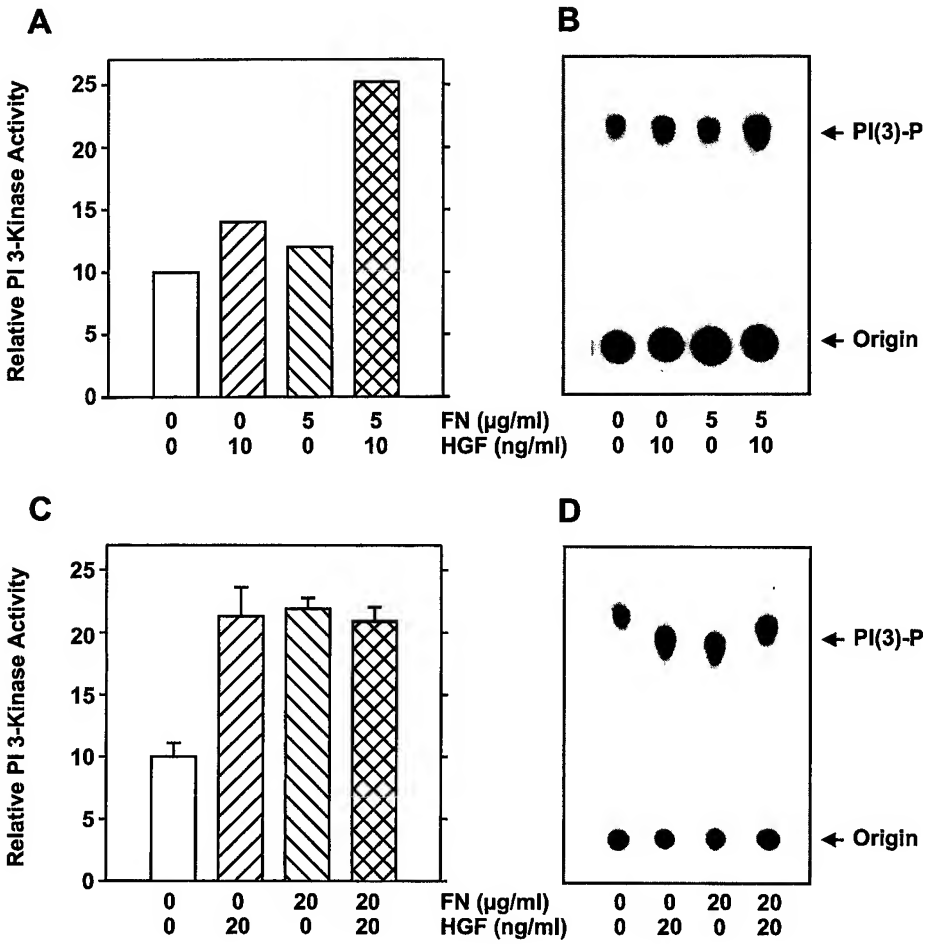

Fig. 4. Cooperative stimulation by HGF and FN of PI 3-kinase activity in detached cells. SP1 cells were serum starved for 24 h and detached. Suspended cells were preincubated in serum-free RPMI 1640 for 15 min and stimulated with HGF, FN, or HGF + FN as indicated for 15 min at 37°C. The cells were then washed and lysed. Clarified cell extracts were normalized for protein and precipitated with anti-phosphotyrosine monoclonal antibody. The immunoprecipitates were washed, and the reaction was initiated by the addition of 20 μg of PI, 30 mM $MgCl_2$, and 25 μCi [γ-^{32}P]ATP. After incubation for 20 min at room temperature, the reaction was stopped by the addition of 100 μl of 1 N HCl. The lipids were extracted by the addition of 200 μl of $CHCl_3/CH_3OH$ (1:1) and were resolved by TLC in a $CHCl_3/CH_3OH/4$ M NH_4OH (9:7:2) solvent mixture. The TLC plate was dried, and migrating lipids corresponding to PI 3-phosphate [*PI(3)-P*] were measured with a PhosphorImager. *A* and *B*, limiting concentrations of HGF (10 ng/ml) and FN (5 μg/ml) were used. This result is representative of two experiments. *C* and *D*, higher concentrations of HGF (20 ng/ml) and FN (20 μg/ml) were used. The mean ± range of duplicates is shown; *bars*, range of duplicates in one experiment.

hibitor PD98059 (44) had no effect on HGF- or FN-induced cell survival or serine phosphorylation of PKB/Akt at concentrations that completely blocked ERK1/2 activation (Fig. 9). In addition, the LY294002 inhibitor caused only a minimal reduction in ERK1/2 activity in SP1 carcinoma cells (Fig. 7*C*). The slight decrease observed could be attributable to a reduction in the basal levels of ERK1/2 activity observed with this drug in other systems (42).

Discussion

Stromal-derived ECM proteins (2–4, 21–23) and growth factors (45–47) provide a balance of signals that regulate cell survival, growth, and differentiation of nonmalignant and malignant epithelium. HGF and other associated growth factors stimulate normal mammary epithelial morphogenesis (reviewed in Ref. 16). However, during tumorigenesis, HGF stimulates phenotypic changes associated with epithelial-mesenchymal transition, invasion, angiogenesis, and metastasis (13–16). HGF has also been shown to be a key survival factor in carcinoma cells (17–19). The mechanisms that regulate the change in HGF response from a morphogenic to a tumorigenic phenotype in epithelial cells are not clear. In the present study, we have examined the cooperative role of FN and HGF in regulating the survival response of carcinoma cells under anchorage-independent conditions that mimic the invasive tumor phenotype.

We reported previously a murine mammary carcinoma cell line, SP1, that expresses HGF and tyrosine-phosphorylated Met (28) in monolayer culture, consistent with the presence of an HGF autocrine loop. Depending on culture conditions, both paracrine and autocrine effects of HGF have been observed in SP1 carcinoma cells. Under serum-starved conditions, paracrine stimulation with exogenous HGF was required for optimal migration through Transwell membranes (38) and colony growth in agar (29). In the present study, we have shown that in the absence of anchorage, serum-starved SP1 cells exhibit reduced tyrosine phosphorylation of Met and undergo apoptotic cell death, whereas the addition of HGF stimulates rephosphorylation of Met at tyrosine residues and increased cell survival. These findings implicate HGF as an important survival factor in carcinoma cells. However, the basal level of Met activation and function may be influenced by extracellular conditions, such as cell adhesion to various substrata (39, 40), cell density effects on autocrine HGF expression and secretion (48), or proteolytic processing of pro-HGF to biologically active forms (49). Our results also showed that the addition of soluble FN promotes survival of detached SP1 cells and shows a cooperative survival effect

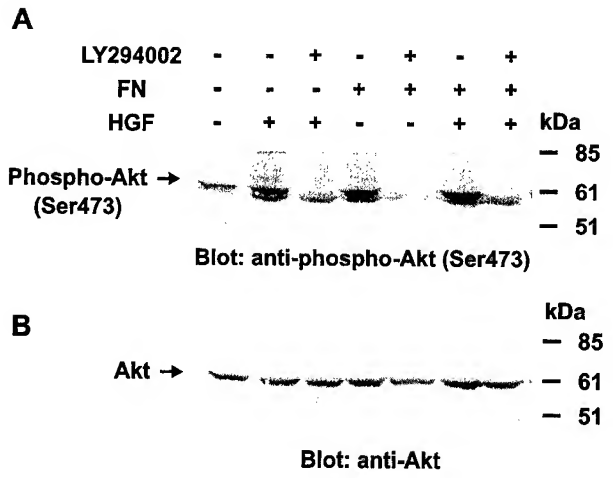

Fig. 5. Cooperative stimulation by HGF and FN of phosphorylation of PKB/Akt at serine 473 in detached SP1 cells. SP1 cells were serum starved overnight, detached, and kept in suspension for 2 h. Cells were treated with HGF (20 ng/ml) or FN (20 μg/ml) as indicated for 40 min. Where LY294002 (20 μM) was used, it was added 10 min prior to HGF and FN treatment. Cells were then lysed, and extracts were analyzed by Western blotting using: *A*, anti-serine 473 phospho-Akt; and *B*, the same blot stripped and reprobed with anti-Akt pan antibody.

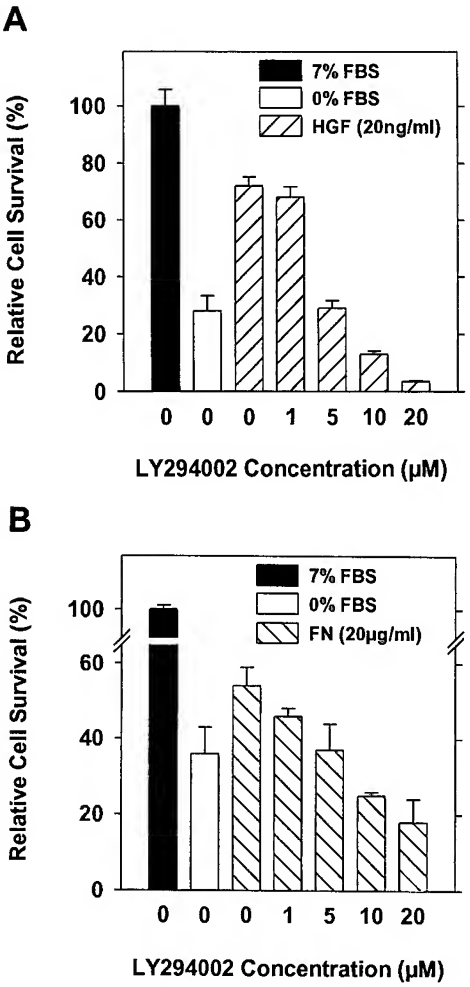

Fig. 6. The PI 3-kinase inhibitor LY294002 blocks HGF- and FN-induced survival of detached SP1 cells. SP1 cells were seeded in suspension cultures without or with the PI 3-kinase inhibitor LY294002 at the indicated concentrations. After 24 h incubation at 37°C, cells were transferred to 96-well plates, and surviving cells were measured with a colorimetric assay as described in "Materials and Methods." *A*, effect of LY294002 on HGF-induced survival. *B*, effect of LY294002 on FN-induced survival. The results are expressed as mean ± range of duplicates and are representative of two experiments; *bars*, range of duplicates in one experiment.

with HGF. The cooperative survival effect in carcinoma cells was most demonstrable at low concentration levels of HGF and FN and may be more relevant to *in vivo* tissue microenvironment. Matrix assembly of FN appears not to be required, because treatment with a M_r 70,000 NH_2-terminal FN fragment, which inhibits FN fibril formation (50), had no effect on FN-induced cell survival (data not shown). In contrast, FN matrix assembly is required for colony growth of carcinoma cells (29). These results further support an antiapoptotic effect of HGF in breast carcinoma cells in the absence of anchorage and suggest that soluble FN can provide signals that enhance the survival effect of HGF. Soluble FN and HGF can also promote a cooperative survival effect in a nonmalignant mammary epithelial cell line, HC11, under detached conditions. However, Met expression is much lower in HC11 compared with SP1 cells (data not shown), whereas HC11 cells show an increased dependence on FN for survival. Thus, soluble FN and HGF may be important in promoting survival of epithelial cells during detachment and dissociation in early-stage carcinogenesis, whereas formation of a FN fibrillar matrix appears to be required for later stages of anchorage-independent growth of carcinoma cells, perhaps as scaffolding for colony formation.

Cell-ECM interactions have been shown to collaborate with growth factor receptors in many biological processes (reviewed in Refs. 24–26), including growth and differentiation of various cell types (24), activation of the Na^+-H^+ antiporter via protein kinase C (51), and activation of downstream signaling molecules, such as MAPK (52) and phospholipase Cγ (53). Our finding that HGF and FN stimulate a strong cooperative survival effect in SP1 cells implies a cooperative interaction between integrin and Met receptors. However, HGF was found to have no effect on expression of β1-integrins or adhesion to FN (data not shown). In addition, FN synthesis is greatly reduced in nonadherent, compared with adherent, SP1 cells (29). Thus, it is unlikely that HGF stimulates survival by up-regulating the FN adhesion system.

Our results further suggest that cell adhesion and spreading on FN substratum promote autoactivation of Met in SP1 cells, whereas sustained activation of Met in nonadherent cells requires paracrine stimulation with exogenous HGF. Similarly, Wang *et al.* (39) showed that cell adhesion elicits activation of Met in melanoma cells. In addition, Sundberg and Rubin (40) showed that stimulation of β1 integrins in fibroblasts induces PDGF-independent tyrosine phosphorylation of PDGF β-receptors. However, unlike adherent carcinoma cells, loss of anchorage has no detectable effect on Met activation in the presence of soluble FN, and anti-HGF neutralizing IgG does not affect FN-induced cell survival (data not shown). Together, these results imply that HGF and

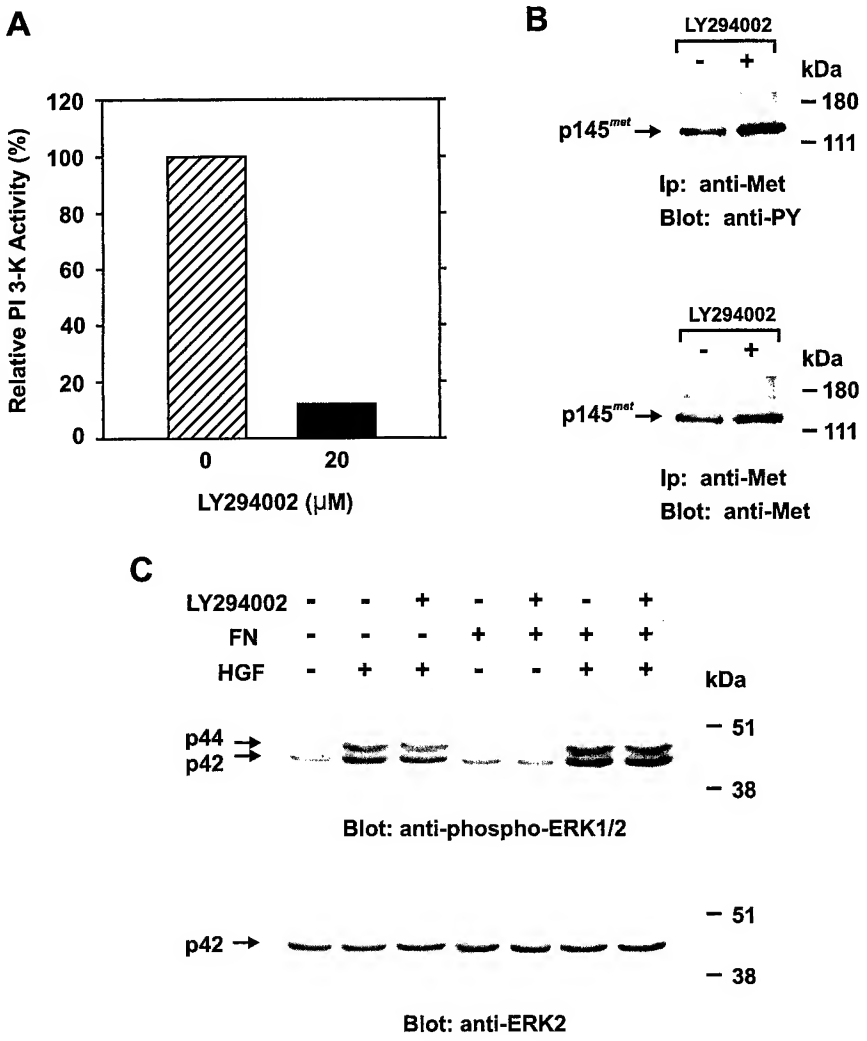

Fig. 7. LY294002 inhibits PI 3-kinase activity without affecting either tyrosine phosphorylation of Met or phosphorylation of MAPK in SP1 cells. SP1 cells were cultured on tissue culture plates in RPMI 1640 with 7% FBS and treated without or with 20 μM LY294002 for 4 h. Cells were assayed for PI 3-kinase activity (*A*) as in Fig. 4 and for tyrosine phosphorylation of Met (*B*) as in Fig. 3. *C*, SP1 cells were prepared and treated as in Fig. 5, and the cell extracts were analyzed by Western blotting with anti-phospho-ERK1/2 antibody. The same blot was stripped and reprobed with anti-ERK2 pan antibody.

FN stimulate cell survival via independent mechanisms, although a common downstream signaling pathway is likely involved in the cooperative effect.

We observed a cooperative increase in PI 3-kinase activity and phosphorylation at serine 473 of PKB/Akt in response to HGF and FN. We also showed that PI 3-kinase activity is required for HGF- and FN-induced PKB/Akt phosphorylation and cell survival. These findings indicate that the PI 3-kinase/Akt pathway is involved in regulating cell survival in this system. PI 3-kinase activity is also required for anchorage-independent colony growth and for HGF-induced proliferation in monolayer culture (33). Activation of PI 3-kinase has been shown to be sufficient for entry into S phase of the cell cycle and in the presence of serum, promotes oncogenic transformation (54). However, because PI 3-kinase is a major regulator of cell survival, the requirement of PI 3-kinase activity for HGF-induced DNA synthesis and colony growth as shown by us (30) and others (31) may be attributable, at least in part, to the role of PI 3-kinase in suppressing apoptosis.

Interestingly, inhibition of c-Src kinase activity by expressing the dominant-negative SRC-RF mutant in SP1 cells has no effect on the survival response to HGF or FN but blocks HGF-induced anchorage-independent growth and cell motility (38). It should be noted that c-Src family tyrosine kinases have been implicated in protection from Fas ligand-induced apoptosis in lymphocytic cells (55), and that expression of v-Src can promote survival in some cell types (43). However, in carcinoma cells, c-Src kinase function appears to be associated primarily with cell adhesion and coactivation of cytoskeletal molecules such as FAK and paxillin (56), which are important in cytoskeletal organization, cell shape, and locomotion. In addition, activation of MEK1/2 and ERK1/2 are not required for HGF- and FN-induced survival responses in detached cells. This observation is distinct from previous reports that cell adhesion and activation of the Ras/MAPK pathway stimulate a PI 3-kinase/Akt-dependent survival response (43). Our results therefore suggest that the PI 3-kinase/Akt pathway is a key effector of HGF- and FN-dependent cell survival of detached carcinoma cells and acts independently of c-Src and MEK1/2, which are involved primarily in cell adhesion-dependent survival and growth responses.

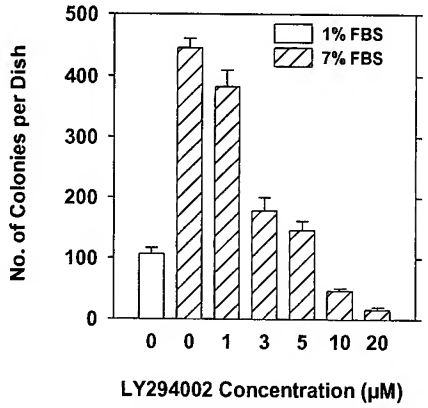

Fig. 8. The PI 3-kinase inhibitor, LY294002, blocks growth of SP1 cells in agar. SP1 cells were cultured (10^3 cells/dish) in 60-mm culture dishes in soft agar (0.36%) with RPMI 1640 with 7% FBS and with the indicated amounts of the PI 3-kinase inhibitor, LY294002. The colony assay was performed as described previously (29). The cultures were incubated for 10 days, and then cells were fixed in methanol and stained with Giemsa. Colonies were counted manually. The results are expressed as the mean number of colonies of quadruplicates and are representative of two experiments; *bars*, SD.

Table 1 c-Src kinase activity does not affect HGF- and FN-induced anchorage-independent survival of SP1 cells

Reagent	Concentration	Relative cell survival (%)[a]		
		SP1	SRC	SRC-RF
–	–	6.1 ± 1.0[b]	7.9 ± 1.0	4.1 ± 1.0
HGF	10 ng/ml	14.3 ± 2.3	12.5 ± 1.6	15.8 ± 3.8
HGF	20 ng/ml	18.2 ± 2.9	18.6 ± 2.4	14.2 ± 3.5
HGF	30 ng/ml	19.2 ± 3.1	20.1 ± 2.5	19.0 ± 4.6
–	–	7.0 ± 1.3	6.5 ± 0.1	7.0 ± 0.1
FN	5 μg/ml	8.8 ± 0.5	9.5 ± 0.1	10.0 ± 0.8
FN	10 μg/ml	15.6 ± 0.2	7.8 ± 0.1	10.0 ± 0.7
FN	20 μg/ml	27.2 ± 4.5	15.0 ± 1.8	21.7 ± 0.8

[a] Serum-starved SP1 cells were incubated in suspension for 24 h with the indicated reagents, and cell survival was assessed with the colorimetric enzyme assay as described in Fig. 6. Relative cell survival was calculated by normalization to results obtained from 7% FBS-treated groups.
[b] Results are expressed as mean ± range of duplicates and are representative of three experiments.

Our demonstration of a cooperative effect of HGF and FN in the activation of the PI 3-kinase/Akt pathway linked to anchorage-independent survival of carcinoma cells is novel. The nature of the cooperation between FN- and HGF-dependent induction of PI 3-kinase activity is not known. Possible mechanisms include increased binding of the p85 subunit to Met or interaction of p85 with signaling molecules associated with the cell adhesion complex (57). FAK is tyrosine phosphorylated in epithelial cells in response to ECM matrix proteins (58) and to HGF in carcinoma cells in monolayer culture (59) and is required for adhesion-dependent cell survival (60). However, soluble FN or HGF has no effect on tyrosine phosphorylation of FAK in detached SP1 cells (data not shown); this observation suggests that FAK is not a key regulator in the present system. This notion is further supported by our finding that c-Src is not involved in the survival response to HGF. We are currently investigating whether ILK, which is activated after cross-linking of β1 integrins in detached cells (61), is involved in the cooperative effect of HGF and FN on PKB/Akt activation and cell survival.

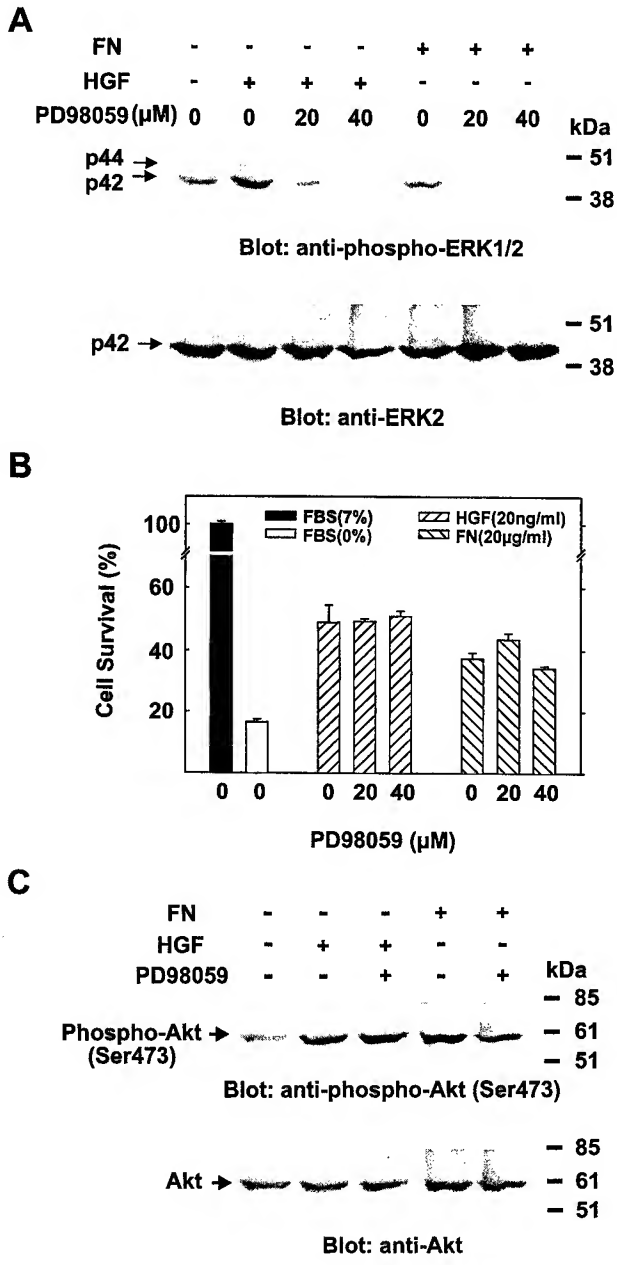

Fig. 9. The MAPK inhibitor PD98059 inhibits HGF-induced phosphorylation of MAPK but does not effect HGF- and FN-induced cell survival and phosphorylation of PKB/Akt at serine 473. *A*, SP1 cells were serum starved overnight, detached, and kept in suspension for 2 h. Cells were treated with HGF (20 ng/ml) or FN (20μg/ml) as indicated for 40 min. Where PD98059 was used, it was added 10 min prior to HGF and FN treatment. Cells were then lysed, and extracts were analyzed by Western blotting with either anti-phospho-ERK1/2 or anti-ERK2 antibody. *B*, SP1 cells were assayed for survival under detached conditions as described in Fig. 6; *bars*, range of duplicates in one experiment. *C*, SP1 cell lysates from *A* were analyzed by Western blotting with anti-serine 473 phospho-Akt antibody. The same blot was stripped and reprobed with anti-Akt pan antibody.

In summary, we have shown a cooperative effect of HGF and FN in anchorage-independent survival of mammary car-

cinoma cells, and that the PI 3-kinase/Akt pathway is a key regulator of this process. These findings are particularly important because they suggest that the PI 3-kinase/Akt signaling pathway is a potential target for inhibiting HGF-induced survival of carcinoma cells during detachment from the primary tumor site and metastasis (62). In addition, cell adhesion enhances Met activation in carcinoma cells, suggesting interaction between integrin- and Met-dependent signaling pathways. Further studies are in progress to determine the role of the cell adhesion complex in HGF-induced survival and growth. Together our results suggest that cooperative signaling via Met and integrin receptors may provide a selective survival advantage in invasive breast cancer.

Materials and Methods

Reagents. PI was purchased from Sigma (Oakville, Ontario, Canada). [γ-^{32}P]ATP and enhanced chemiluminescence (ECL) reagents were purchased from DuPont NEN Life Science Products (Boston, MA). Rabbit antirat PI 3-kinase IgG (specific for the p85 subunit) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit antimouse Met and anti-ERK2 IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PI 3-kinase inhibitor LY294002 (41) and MEK1/2 inhibitor, PD98059 (44), were purchased from Calbiochem (San Diego, CA). Rabbit anti-phospho-Akt (ser473) and pan Akt antibodies were from New England Biolabs (Beverly, MA). The phosphospecific ERK1/2 antibody was a gift from Erik Schaefer (BioSource International, Camarillo, CA).

Tissue Culture and Cell Lines. The SP1 tumor is a spontaneous nonmetastatic murine mammary intraductal adenocarcinoma isolated from an 18-month-old CBA/J female retired breeder in the mouse colony at Queen's University (63, 64). The established SP1 cell line was frozen at −70°C to maintain stocks. HC11 murine mammary carcinoma cells were obtained from Dr. D. Medina (Baylor College of Medicine, Houston, TX). Maintenance medium for SP1 cells was RPMI 1640 (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 7% FBS (Life Technologies). HC11 cells were cultured in RPMI 1640 supplemented with 10% FBS, 5 μg/ml insulin (Life Technologies), and 10 ng/ml EGF (Sigma). Cells were kept in culture for no more than 3 months before thawing a fresh stock and were tested periodically for *Mycoplasma*.

Transfection of Wild-Type and Mutant c-Src cDNAs. cDNAs encoding wild-type c-*src* (SRC) and a dominant-negative double mutant of c-*src* (SRC-RF), with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F), ligated into the pRc/CMV plasmid (Invitrogen, San Diego, CA) carrying the neomycin resistance marker, were obtained from Dr. J. Brugge (Department of Cell Biology, Harvard Medical School, Boston, MA; Ref. 65). SP1 cells expressing mutant c-Src and wild-type c-Src were established using the stable transfection lipofectAMINE method as described previously (38). Pooled transfected cells were selected with G418 (450 μg/ml). We showed previously that cells transfected with the dominant-negative SRC-RF mutant showed a 4-fold reduction in c-Src kinase activity compared with SRC-transfected cells, whereas the levels of Met protein or activity and downstream signaling (*e.g.*, phospholipase Cγ activity) were unaffected (38).

Apoptosis Assay. The *in situ* end-labeling procedure described previously (66) was used to detect DNA fragmentation in carcinoma cells. Paraformaldehyde-fixed cells on glass slides were immersed in 0.1 M PBS for 15 min and in buffer A [50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.005% BSA (Sigma), pH 7.5] for an additional 15 min, all at room temperature. The cells were then incubated for 70 min at 37°C in a humidified chamber in a solution of buffer A containing 0.01 mM each of dATP, dCTP, dGTP, biotin-16-dUTP (Boehringer Mannheim, Laval, Quebec, Canada), and 20 units/ml *Escherichia coli* DNA polymerase I (Promega, Madison, NY). As negative controls, the biotinylated UTP or DNA polymerase I was omitted from the above incubating solution for some groups. The reaction was terminated by two 15-min washes in 0.1 M PBS, 0.05% Tween 20 at 4°C. The cells were then incubated in premixed Vectastain avidin and biotinylated horseradish peroxidase complex (ABC; Vector Laboratories, Inc.; 1:100) for 2 h at room temperature, followed by three 15-min washes in 0.1 M PBS, 0.05% Tween 20. Staining was then developed with 0.025% diaminobenzidine and 0.05% H_2O_2 in 0.1 M PBS for 12 min at room temperature. The slides were air-dried overnight, and the cells were then lightly counterstained with hematoxylin and coverslipped in Permount (Fisher Scientific, Nepean, Ontario, Canada).

Survival Assay. Prestarved SP1 cells were seeded at a density of 2×10^4 cells in 1.5 ml of RPMI 1640 containing 0.5 mg/ml BSA and reagents as indicated into 0.6% agar-coated, 35-mm Corning non-tissue culture plates. After 24 h incubation at 37°C, the cells were collected and centrifuged in Eppendorf tubes (1000 rpm for 5 min) and stained for live/dead cells with a 1:1 mixture of acridine orange (Sigma) and ethidium bromide (Sigma), each at 4 μg/ml (67). A Leitz fluorescence microscope equipped with epi-illumination was used to count live/dead cells. Nuclei of viable cells stained uniformly green with acridine orange, which intercalates with DNA. Early apoptotic cells, in which membranes are still intact, stained green with patches of chromatin condensation in nuclei but excluded ethidium bromide. Late apoptotic cells, in which membranes are disrupted, stained red with ethidium bromide, also with patches of condensed chromatin in nuclei. In a parallel analysis, an enzyme survival assay, as described by Khwaja *et al.* (43), was also carried out. For the enzyme assay, cells were replated into a 96-well plate with 7% FBS/RPMI medium and incubated at 37°C for 4 h. A colorimetric method based on the conversion of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium] to formazan (CellTiter aqueous kit; Promega Corp., Madison, WI) was used to measure cell survival.

Colony Assay. Colony assays were performed as described previously by Saulnier *et al.* (29). Briefly, a solution of 1.2% Bactoagar (Difco Lab) was mixed (1:1) with 2× RPMI 1640, supplemented with 7% FBS, and layered onto 60-mm tissue culture plates and allowed to solidify. SP1 cells (10^3/2.5 ml) were mixed in a 0.36% Bactoagar solution prepared in a similar way and layered (2.5 ml/plate) on top of the 0.6% Bactoagar. Plates were incubated at 37°C in 5% CO_2 for 8–10 days. Colonies were fixed with 100% methanol, stained with Giemsa (4%, v/v; BDH, VWR Scientific, Mississauga, Ontario, Canada), and counted manually.

Immunoprecipitation and Western Blotting. SP1 cells were grown to confluence and serum starved for 24 h. Cells were rinsed with cold PBS buffer and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM Na_3VO_4, 50 mM NaF, 1 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. The supernatants were measured for protein content with a bicinchoninic acid protein assay (Pierce, Rockford, IL), and were adjusted to equal protein concentrations. Equal volumes of each supernatant were incubated with appropriate mouse or rabbit antibodies for 1 h. Immunoprecipitates were collected on protein A-Sepharose (Amersham-Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) for 1 h, washed three times with lysis buffer, separated on 8% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane by electroelution. The membrane was blocked with 3% skimmed milk or 1% BSA in TBST buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 15 min and probed with the appropriate primary antibodies for 1 h at room temperature or overnight at 4°C. The membrane was washed three times with TBST for 5 min each, incubated with horseradish peroxidase-labeled secondary donkey antirabbit IgG (Amersham, Oakville, Ontario, Canada) or sheep antimouse IgG (Amersham) for 15 min, and washed three times for 10 min each with TBST. Immune complexes were detected with ECL.

PI 3-Kinase Assay. PI 3-kinase assays were performed essentially as described previously (30, 33). In brief, approximately 1×10^6 SP1 cells were seeded in 10-cm plates and serum starved for 24 h. Cells were detached by incubation with PBS containing 0.5 mM EDTA and 0.5 mM EGTA for 5 min at 37°C. Suspended cells were preincubated at 37°C for 15 min to stabilize baseline activity and stimulated by the addition of reagents as indicated. The cells were then washed with PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and lysed in cold lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.0), 0.92 mM $CaCl_2$, 0.49 mM $MgCl_2$, 10% glycerol, 1% NP40, 100 μM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. Clarified cell extracts were normalized for protein concentration and precipitated with anti-phosphotyrosine monoclonal antibody. In some experiments, anti-PI 3-

kinase IgG was used with similar results. The immunoprecipitates were washed two times with PBS/1% NP40, two times with PBS, two times with 0.1 M Tris (pH 7.0) and 0.5 M LiCl, once with TNE [10 mM Tris (pH 7.4), 100 mM NaCl, and 1 mM EDTA], and once with 20 mM HEPES (pH 7.4). Immune complexes were suspended in 50 μl of 20 mM HEPES (pH 7.4) with 20 μg of sonicated PI and were incubated on ice for 10 min. The reaction was initiated by addition of 30 mM $MgCl_2$ and 25 μCi [γ-^{32}P]ATP. After incubation for 20 min at room temperature, reactions were stopped by the addition of 100 μl of 1 N HCl, and the lipids were extracted by addition of 200 μl of $CHCl_3/CH_3OH$ (1:1) and were resolved by silica gel plate (Whatman Ltd., Maidstone, England) chromatography in $CHCl_3/CH_3OH/4$ M NH_4OH (9:7:2) solvent. The TLC plate was dried, and labeled lipids migrating as phosphatidylinositol 3-phosphate were measured with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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